

Posttranslational Protein Modification in *Archaea*

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INTRODUCTION

With complete genome sequences appearing at an ever more rapid rate, attention is becoming increasingly directed towards describing the protein complement of a given organism, i.e., the proteome. Studies of proteins conducted both at the level of the individual polypeptide and cellwide have revealed that the repertoire of expressed proteins can expand beyond what is predicted by direct translation of the complement of open reading frames contained within a genome. For example, the proteome can assume additional levels of complexity with differential expression of individual polypeptides or members of protein families as a function of developmental stage or in response to environmental cues. The various permutations of protein-protein interactions possible further expand the complexity of the proteome. However, one of the most important and fundamental aspects of proteomic complexity comes from the various processing events that many proteins experience following their synthesis, i.e., posttranslational modification.

Proteins can be modified posttranslationally by covalent attachment of one or more of several classes of molecules, by the formation of intra- or intermolecular linkages, by proteolytic processing of the newly synthesized polypeptide chain, or by any combination of these events. By chemically linking various modifying groups either permanently or temporarily and by allowing for changes in the molecular composition of the modifying moieties, covalent modifications can endow proteins with properties that are very different from those that are predicted by the encoding genes. Examples of such covalent modifications include glycosylation, lipid attachment, phosphorylation, and methylation.

The covalent bonding of pairs of Cys residues to form disulfide bridges not only modulates the three-dimensional conformation of a polypeptide chain but can also be used to maintain proteins in multisubunit complexes. Controlled reduction and reoxidation of protein disulfide bonds is also employed in electron transfer reactions fundamental to many cellular processes. Proteolytic processing of newly synthesized polypeptide chains similarly allows the cell to control the folding and function of a protein. By removing specific targeting sequences or other stretches of amino acid residues, the cell is able to control where, when, and how a protein will act. As such, posttranslational modifications can significantly modulate the physicochemical and biological properties of a protein through effects on protein function, subcellular localization, oligomerization, folding, or turnover. The distribution of posttranslational mod-

ifications and their effects on protein chemistry and cell biology become even broader when one also considers the effects of additional, secondary posttranslational modification steps such as the addition of organic (e.g., flavins) or inorganic (e.g., metal groups) cofactors. Such modifications, however, lie beyond the scope of this review.

Long-known to be widespread in *Eucarya* and *Bacteria*, it is becoming clear that posttranslational modification of proteins also takes place in *Archaea*. Best known in their capacities as extremophiles, i.e., microorganisms able to thrive in the harshest environmental conditions on this planet, *Archaea* express proteins that enable them to succeed in such habitats. Indeed, archaeal proteins are able to remain properly folded and functional in the face of extremes of salinity, temperature, and other adverse physical conditions that would normally lead to protein denaturation, loss of solubility, and aggregation. Although posttranslational modifications may help archaeal proteins overcome the challenges presented by their surroundings, in most cases, the reason for posttranslational modification of a particular archaeal protein remains unclear. Table 1 lists the posttranslational modifications that archaeal proteins may experience.

Analysis of the various posttranslational modifications experienced by archaeal proteins has served to reveal not only novel protein modifications not previously observed in *Eucarya* or *Bacteria* but also variations of previously characterized posttranslational modifications. By and large, however, archaeal

TABLE 1. Posttranslational modifications of archaeal proteins

Posttranslational modification	Comment
Glycosylation	N-glycosylation, O-glycosylation
Lipid modification	Lipoproteins, isoprenylation, acylation, GPI anchoring
Phosphorylation	Phosphoaspartate, phosphohistidine, phosphoserine, phosphothreonine, phosphotyrosine
Disulfide bonds	Cytosolic proteins
Proteolytic processing	Signal sequence cleavage, intein excision, amino-terminal and carboxy-terminal maturation
Methylation	Methylarginine, methylaspartic acid, methylcysteine, methylglutamic acid, methylglutamine, methylhistidine, methyllysine
Acetylation	
Amino acid modification	Hypusination, thiolation

posttranslational modifications often resemble their eucaryal or bacterial counterparts. Hence, elucidating such similarities provides insight into evolutionary relationships across the three domains of life. Moreover, the mosaic profile of eucaryal, bacterial, and archaeal traits that describes posttranslational protein modification in *Archaea* also holds true when one examines the enzymes and mechanistic steps involved in archaeal protein modification processes. Here too, examination of archaeal systems has served to expand our understanding of natural pathways or to underscore the similarities between archaeal, eucaryal, and/or bacterial biology. Nonetheless, numerous aspects of archaeal posttranslational processing remain poorly described. In the following review, what is currently known of posttranslational protein modification in *Archaea* is considered.

PROTEIN GLYCOSYLATION

One of the more prevalent posttranslational modifications experienced by eucaryal proteins is glycosylation. Indeed, protein glycosylation, which begins in the lumen of the endoplasmic reticulum and continues in the Golgi apparatus, is thought to be experienced by more than half of all eucaryal proteins (12). Upon translocation into the endoplasmic reticulum, proteins can be N-glycosylated, when branched oligosaccharide trees of 14 subunits are initially added to selected Asn residues. O-glycosylation of Ser or Thr residues usually takes place in the Golgi. In *Eucarya*, the glycan moieties of glycosylated proteins fulfill a multitude of roles related to protein solubility, folding, stability and turnover, and subcellular localization as well as participating in numerous recognition events (46, 157, 333, 409, 448). Long believed to be an exclusively eucaryal trait, it is now clear that both *Bacteria* and *Archaea* are also capable of attaching glycan moieties to selected proteins (285, 292, 381, 425, 445, 456). A list of those archaeal strains reported to contain glycosylated proteins is provided in Table 2.

Glycosylated Archaeal Proteins

S-layer glycoproteins. The surface (S)-layer glycoprotein of the halophilic archaeon *Halobacterium salinarum* was the first prokaryotic glycoprotein to be described in detail (246, 283). Subsequently, S-layer glycoproteins have been studied in numerous prokaryotes (292, 381–383). Serving as the main, if not sole, component of the protein layer surrounding many archaeal cells (101, 382, 383) (Fig. 1), S-layer glycoproteins remain among the best-characterized archaeal glycoproteins. Indeed, examination of the processes used for glycosylation of archaeal S-layer glycoproteins has not only served to enhance our understanding of prokaryotic cell surface biogenesis but has also provided insight into the general phenomenon of protein glycosylation in *Archaea*.

While the glycosylated nature of S-layer proteins has been proposed in many archaeal species, experimental proof for this posttranslational modification is limited to the S-layer glycoproteins of *Halobacterium salinarum* (246), *Haloferax volcanii* (421), *Haloarcula japonica* (299), *Methanothermobacter fervidus* (204), *Methanothermobacter sociabilis* (41), *Sulfolobus* spp. (146), and components of the S-layer of *Staphylothermus marinus*

TABLE 2. Archaeal species reported to contain glycoproteins

Species	Evidence for glycosylation ^a	Reference(s)
<i>Haloarcula japonica</i>	G	299
<i>Haloarcula marismortui</i>	C	136
<i>Halobacterium saccharovorum</i>	E	389
<i>Halobacterium salinarum</i>	A, B	246, 280
<i>Haloferax mediterranei</i>	E	232
<i>Haloferax volcanii</i>	A, B, D	98, 421
<i>Methanobacterium bryantii</i>	D, F	219
<i>Methanococcus deltae</i>	F, H	27
<i>Methanococcus mazei</i>	D	481
<i>Methanococcus voltae</i>	A, B	453
<i>Methanosaeta soehngenii</i>	A	340
<i>Methanospirillum hungatei</i>	E, F	406
<i>Methanothermobacter fervidus</i>	A, B, E	196, 204
<i>Methanothermobacter sociabilis</i>	A, B	41
<i>Natrialba magadii</i>	E	197
<i>Pyrococcus furiosus</i>	C, D, E	44, 230, 231, 455, 464
<i>Sulfolobus acidocaldarius</i>	A, B, E, G, H	146, 147, 161, 258, 286
<i>Sulfolobus shibatae</i>	E	112
<i>Sulfolobus solfataricus</i>	D, E	106, 146, 262
<i>Staphylothermus marinus</i>	C, F	345
<i>Thermococcus litoralis</i>	E	44, 145
<i>Thermococcus stetteri</i>	E	184
<i>Thermoplasma acidophilum</i>	A, B	478
<i>Thermoplasma volcanium</i>	E	112

^a A, saccharide-amino acid linkage determined; B, glycan structure determined; C, saccharide content determined; D, lectin binding; E, glycan staining; F, deglycosylation; G, inhibition of glycosylation; H, aberrant SDS-PAGE migration. Where A and/or B is true for a given glycoprotein, other evidence for glycosylation is not listed for that case. See text for details.

(345). Although the experimental evidence for glycosylation, ranging from chemical characterization of the bound glycan moieties to glycol staining, is stronger in some cases than others, it has been calculated that these S-layer glycoproteins experience an overall degree of glycosylation of up to 15% (292, 382).

Like eucaryal glycoproteins, archaeal S-layer glycoproteins can undergo both N- and O-glycosylation. In contrast, bacterial S-layer glycoproteins contain only O-linked glycans (285, 445), although examples of N-glycosylation of other bacterial proteins have been shown (107, 425, 456). Analysis of the composition of the N-linked glycan moieties of archaeal S-layer glycoproteins has revealed the wide variety of saccharides available for protein glycosylation in *Archaea*, including galactofuranose, galactouronic acid, glucose, glucuronic acid, iduronic acid, mannose, N-acetylgalactosamine, N-acetylglucosamine, and rhamnose (204, 280, 335, 421, 456). In many cases, these sugar subunits may themselves be modified by methylation or sulfation. Such diversity in the range of saccharides used in archaeal S-layer glycoprotein N-glycosylation exceeds that seen in the bacterial and eucaryal N-glycosylation processes (425, 456).

(i) S-layer glycoproteins reveal unique aspects of archaeal protein glycosylation. Despite the proposed evolution of the eucaryal N-glycosylation system from a precursor process in *Archaea* (46, 157), studies of archaeal S-layer glycoprotein glycosylation, and in particular glycosylation of the *Halobacterium salinarum* S-layer glycoprotein, have revealed differences in

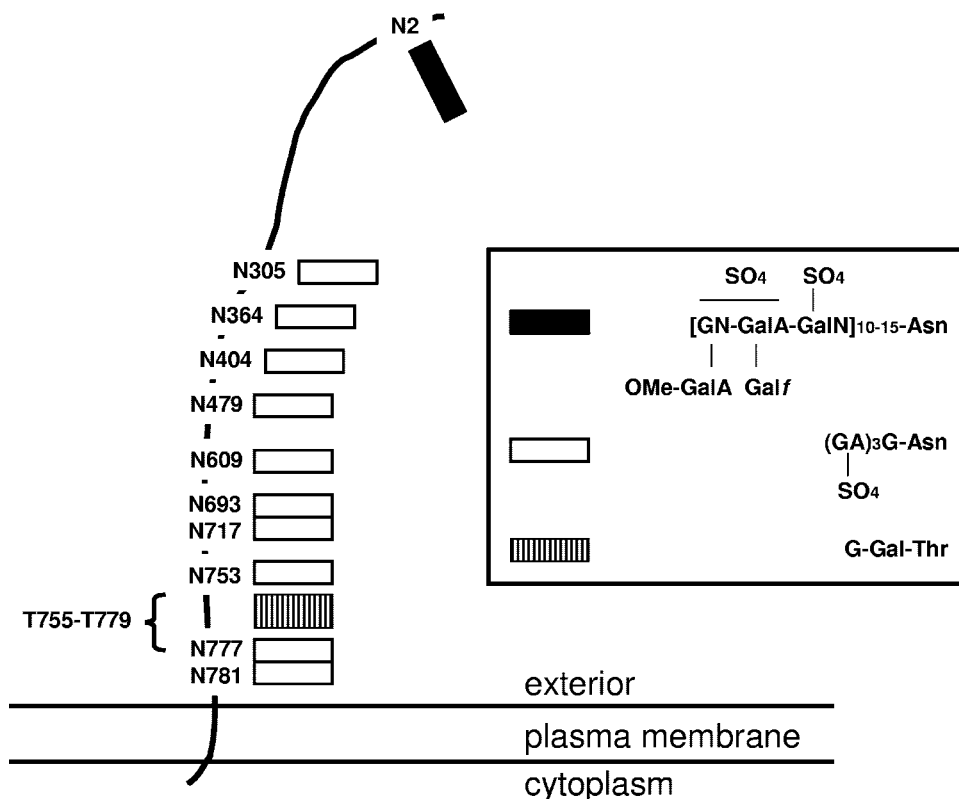


FIG. 1. Schematic depiction of the glycosylation of the *Halobacterium salinarum* S-layer glycoprotein. The topology of the S-layer glycoprotein, the positions of the 11 Asn residues that undergo N-glycosylation, and the heavily O-glycosylated Thr-rich region between Thr-755 and Thr-779 are indicated (246). The inset shows the composition of the three oligosaccharide moieties bound to the protein (247). Abbreviations used: G, glucose; GA, glucuronic acid; Gal, galactose; GalA, galacturonic acid; Galf, galactofuranose; GalN, *N*-acetylgalactosamine; GN, *N*-acetylglucosamine; OMe, *O*-methyl; SO₄, sulfate. Approximately a third of the glucuronic acid residues may be replaced by iduronic acid.

N-glycosylation in the two domains. Such differences are reflected, for example, in the failure thus far to detect antennary structures in *Archaea* similar to those employed in eucaryal protein N-glycosylation (46, 157, 235, 333, 409, 442), or in the identified amino acid sequence motifs recognized by the archaeal N-glycosylation machinery.

It was observed that replacement of the Ser residue of the Asn-2-Ala-3-Ser-4 sequence of the *Halobacterium salinarum* S-layer glycoprotein with Val, Leu, or Asn did not prevent N-glycosylation at the Asn-2 position (486). By contrast, the eucaryal system almost invariably recognizes the Asn-X-Ser/Thr sequence motif, where X is any residue apart from Pro (46, 157, 235, 333, 409, 442), although a rare exception of N-glycosylation at an Asn-Gly-Gly-Thr motif has been reported (211). The ability of *Archaea* to glycosylate proteins at Asn residues that are not part of the consensus Asn-X-Ser/Thr motif suggests that predictions of the glycosylation status of archaeal proteins may have overlooked similar or novel N-glycosylation sites. Moreover, the finding that the repeating sulfated pentasaccharide moiety attached at the Asn-2 position of the *Halobacterium salinarum* S-layer glycoprotein through an *N*-acetylgalactosamine link is chemically distinct from the sulfated polysaccharide unit attached via glucose subunits found at the other ten N-glycosylation sites in the S-layer glycoprotein (247) implies the existence of two different *N*-saccharyl-

transferases in this species. At present, it remains unclear how the cell would distinguish between the different N-glycosylation sites.

Finally, the linkage of glycan moieties to the *Halobacterium salinarum* S-layer glycoprotein at selected Asn residues through either *N*-acetylgalactosamine or glucose subunits (335) is in contrast to the *N*-acetylglucosamine linkage largely employed in eucaryal N-glycosylation (46, 157, 235, 333, 409, 442). In the case of the eucaryal protein laminin, however, N-glycosylation involves a β -glucosyl-Asn protein linkage (385). It is of note that laminin is a component of the extracellular basement membrane, a structural layer surrounding mammalian cells in a manner reminiscent of the archaeal S-layer.

In addition to N-glycosylation, archaeal S-layer glycoproteins can also be modified by O-glycosylation of selected Ser or Thr residues. In both *Halobacterium salinarum* and *Haloferax volcanii*, Thr-rich regions adjacent to the membrane-spanning domain of the protein are decorated at numerous positions with galactose-glucose disaccharides (283, 421). Interestingly, a glycoprotein isolated from a eucaryal basement membrane contains a similar disaccharide (254). Presently, little is known of the steps involved in archaeal O-glycosylation or the relation of such steps to the parallel eucaryal or bacterial processes.

Flagellins. In *Archaea*, cell motility mediated by flagella has been reported for representatives of the major phenotypic groups, i.e., the halophiles, the methanogens, the thermophiles, and the hyperthermophiles, largely based on microscopic investigation (20, 184, 436). Although fulfilling similar roles, archaeal flagella bear little resemblance to their better-characterized bacterial counterparts (7, 265) in terms of structure or assembly. Such differences become evident when one considers the flagellar filament in the two domains. Ultrastructural studies have shown that, unlike bacterial filaments, archaeal flagellar filaments are not hollow structures (72) and that the archaeal structures are generally thinner than their bacterial counterparts (79, 185, 190, 406).

Archaeal and bacterial flagella also differ at the level of flagellin, the major structural component of the flagellar filament. Whereas bacterial flagella are, for the most part, composed of a single type of flagellin, archaeal flagellar filaments are made up of several types of flagellins (with the possible exception of *Sulfolobus solfataricus*, where genome annotation efforts have reported the existence of only a single flagellin-encoding gene) (20, 184, 436). Indeed, archaeal and bacterial flagellins do not share sequence similarity (19). Moreover, many archaeal flagellins are glycosylated (184), a posttranslational modification that is considered rare for bacterial flagellins (95, 139, 291, 384, 435).

(i) Evidence for flagellin glycosylation. Glycosylation has been reported for flagellins of numerous archaeal strains (112, 184, 196, 197, 389, 436), including *Halobacterium salinarum* (470), *Methanococcus deltae* (27), *Methanococcus voltae* (453), and *Methanospirillum hungatei* (406). In most of these examples, the evidence for glycosylation comes from studies employing glycan-detecting stains, such as thymol-sulfuric acid or periodic acid-Schiff reagent. Such techniques, however, may not always accurately reflect the glycosylated nature of a protein (222). Hence, additional evidence for glycosylation is desirable.

This has been achieved for the flagellins of *Halobacterium salinarum* and *Methanococcus voltae*, for which the chemical compositions of the covalently linked glycan moieties have been elucidated. The *Halobacterium salinarum* flagellin contains a sulfated glycoconjugate, N-linked through a glucose bridge and based on glucuronic or iduronic acid, similar to the glycan moiety found on the S-layer glycoprotein (420, 468). More recently, *Methanococcus voltae* flagellins have been shown to contain a novel N-linked trisaccharide (453), despite the fact that earlier glycoprotein staining-based studies had failed to detect flagellar glycosylation in this species (195). Analysis of trypsin-generated peptides derived from the *Methanococcus voltae* S-layer glycoprotein also revealed modification by the same novel trisaccharide (453), suggesting a common glycosylation process for the two proteins. Support for the glycosylation of *Methanospirillum hungatei* flagella beyond glycan staining was presented by chemical deglycosylation with trifluoromethanesulfonic acid, a treatment that decreased molecular mass, as estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (406). The same was noted for *Halobacterium salinarum* flagellins upon similar treatment (247).

The glycosylated nature of *Methanococcus deltae* flagellins was indicated upon incubation of cultures with bacitracin, an

antibiotic that interferes with protein glycosylation (see below) (247). Such treatment resulted in more rapid migration of the protein as reflected by SDS-PAGE analysis (27). Similar bacitracin treatment, however, had no effect on the glycosylation of *Halobacterium salinarum* flagellins, as gauged by migration in SDS-PAGE, although incubation with EDTA, thought to specifically inhibit an externally oriented Mg^{2+} -dependent oligosaccharidetransferase (420), successfully modified flagellin migration. By contrast, treating cells with EDTA did lead to the appearance of *Methanococcus deltae* flagellins of lower apparent molecular weight (27). Together, these observations point to differences in the glycosylation machineries of the two species.

Other proteins. While the bulk of attention on archaeal protein glycosylation has focused on S-layer glycoproteins and flagellins, other archaeal glycoproteins have been identified. Of those additional glycoproteins whose identities are known, the majority are membrane associated. In many instances, these are binding proteins involved in nutrient uptake (see below), such as the maltose/trehalose-binding proteins of *Thermococcus litoralis*, shown to react with glyco-stain (145) and of *Pyrococcus furiosus*, shown to contain glucose-containing glycan moieties by lectin binding and molecular analysis (231), or the *Pyrococcus furiosus* cellobiose-binding protein, which reacts with lectins and glyco-stain (230). Glyco-staining also indicated the glycosylated nature of *Pyrococcus furiosus* CipA and CipB, two ABC transporter binding proteins whose expression is up-regulated in response to cold shock in this hyperthermophile (464). Glycosylation of pyrolysin, a thermostable serine-protease also associated with *Pyrococcus furiosus* membranes, was proposed on the basis of sequence analysis that revealed the presence of numerous potential N-glycosylation sites and supported by glyco-staining of the protein (455).

Based on lectin binding, a series of glycosylated sugar-binding proteins, apparently containing mannose, glucose, galactose, and N-acetylglucosamine, was detected in *Sulfolobus solfataricus* membranes (106). *Sulfolobus acidocaldarius* cytochrome *b*_{558/566} was shown to be a highly glycosylated integral membrane protein, containing both O-linked mannose subunits and N-linked hexasaccharides (161). Analysis of the composition of the latter glycan moiety revealed the presence of glucose, mannose, and N-acetylglucosamine in addition to 6-sulfoquinovose (484). 6-Sulfoquinovose (or 6-deoxy-6-sulfo-glucose) is a rare acidic sugar, commonly found in the glycolipids of chloroplasts and photosynthetic bacteria (177), but not previously found in a glycoprotein. The glycosylated character of a membrane-associated *Sulfolobus solfataricus* protein serine/threonine kinase was confirmed through precipitation of a protein with kinase activity using lectin-conjugated agarose beads and by the decreased apparent molecular mass of the protein and resistance to glyco-staining following treatment with chemical deglycosylation agents (262).

In addition to membrane proteins, secreted archaeal glycoproteins have also been detected. Lectin binding and chemical deglycosylation confirmed the glycosylated nature of the copper response extracellular proteins secreted by the copper-resistant methanogen *Methanobacterium bryantii* BKYH (219). Indeed, differential glycosylation is responsible for the appearance of multiple isoforms of the copper response protein. A secreted, inducible alkaline phosphatase purified from *Haloar-*

TABLE 3. N-glycosylation of proteins across the three domains of life^a

Parameter	Eukarya	Archaea	Bacteria (<i>Campylobacter jejuni</i>)
Site	ER (Golgi)	Plasma membrane	Plasma membrane
Saccharide donors	UDP-GlcNAc, GDP-Man, dolicholphosphate-Man/Glc	UDP-saccharide, GDP-Man?, dolicholphosphate-Man/Glc?	UDP-saccharide
Lipid carrier	Dolicholpyrophosphate	Dolicholphosphate, dolicholpyrophosphate	Undecaprenolpyrophosphate
Addition of saccharides following lipid flipping	Yes	No	No
Modification of lipid-bound oligosaccharide	No	Yes	Yes
Final oligosaccharide composition	GlcNAc ₂ Man ₉ Glc ₃	Variable	GalNAc ₂ (Glc)GalNAc ₃ Bac
Protein glycosylation motif	Asp-X-Ser/Thr	Asp-X-Ser/Thr/Val/Leu/Asp	Asp-X-Ser/Thr
Linking sugar	GlcNAc	Variable	GalNAc
Oligosaccharide-transferring enzyme	Oligosaccharide transferase complex	STT3 (isoforms?), additional proteins?	PglB
Oligosaccharide modification following protein transfer	Yes	?	No

^a Abbreviations used: Bac, bacillosamine; Glc, glucose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Man, mannose; ER, endoplasmic reticulum.

cula marismortui was shown to be glycosylated, in part through the use of radiolabeled glucosamine-containing growth medium (136). Quantitative analysis revealed that glycosylation accounted for 3% of the mass of the protein. Based on glyco-staining, a secreted enzyme possessing thermostable amylopullulanase activity, i.e., capable of hydrolyzing both α -1,6 linkages in pullulan and α -1,4 linkages in amylose and soluble starch, was detected in the growth media of both *Pyrococcus furiosus* and *Thermococcus litoralis* (44). Based on aberrant SDS-PAGE migration and sequencing data, it has been proposed that the partially secreted acid protease of *Sulfolobus acidocaldarius*, thermopisin, is also glycosylated (258).

In addition to these identified membrane and secretory glycoproteins, numerous other glycoproteins, uncharacterized apart from their glycosylated nature, have been reported. Using lectin-based purification techniques, a 152-kDa glycoprotein was isolated from *Thermoplasma acidophilum* membranes (478). Subsequent analysis of the glycan moiety of the protein revealed it to be a highly branched, mannose-based structure, N-linked to the polypeptide chain through an *N*-acetylglucosamine subunit. Several lectin-binding proteins have been observed in *Methanococcus mazei* S-6, with the levels of these glycoproteins related to the adoption of morphologically distinct forms by the cells (481). In *Haloferax volcanii*, membrane glycoproteins of 150, 98, 58, and 54 kDa, distinct from the S-layer glycoprotein, were identified in lectin-based studies (98). A second study of the same strain noted the presence of glycoproteins of 105, 56, and 52 kDa in whole-cell lysates (489). It remains to be seen whether any of the proteins identified in the two studies are the same and whether the smaller glycoproteins are derived from the heavier polypeptides.

Relying on glyco-staining, lectin-binding techniques, and treatments with inhibitors of glycosylation or deglycosylating agents, the membranes of both *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* were shown to contain unidentified glycoproteins distinct from the S-layer glycoprotein (147, 262). Glycoprotein staining was used to identify a series of glycosylated proteins in *Pyrococcus furiosus* membranes that are dis-

tinct from CipA and CipB and the expression of which is related to growth temperature (464).

Process of Protein N-Glycosylation in Archaea

In *Eucarya*, N-glycosylation begins on the cytoplasmic face of the endoplasmic reticulum membrane, where nucleotide-activated monosaccharides are sequentially added by membrane-embedded monosaccharyltransferases to the saturated polyisoprenol-based lipid carrier dolichol pyrophosphate. This generates the heptasaccharide core of the glycan structure initially found on all eucaryal N-glycosylated proteins (46, 157, 235, 333, 409, 442). Once assembled, the glycan-charged lipid translocates (or "flips") across the plane of the endoplasmic reticulum membrane bilayer so that the oligosaccharide is now oriented within the endoplasmic reticulum lumen. The translocation of the glycan-charged dolichol pyrophosphate across the membrane is catalyzed by an ATP-independent flippase (165), identified as the RTF1 protein in *Saccharomyces cerevisiae* (159), with homologues reported in other *Eucarya* (158). Additional sugar subunits are then added to the lipid-bound polysaccharide, transferred from flipped, lumen-facing dolichol phosphate glucose or mannose carriers (158). The completed oligosaccharide is next transferred to appropriate Asn residues of a nascent polypeptide chain entering the endoplasmic reticulum (46, 157, 235, 333, 409, 442). This is mediated by oligosaccharide transferase, a multisubunit complex associated with the translocon, the membrane protein complex responsible for protein translocation across the endoplasmic reticulum membrane (392).

If, as proposed (46, 157), the elaborate process responsible for protein N-glycosylation in *Eucarya* originated from a simpler archaeal system, then many of the fundamental steps and central components involved in eucaryal protein N-glycosylation should also be present in *Archaea*. As summarized in Table 3 and discussed in the following section, available evidence suggests that this is indeed the case.

Dolichol carrier. Across evolution, isoprene-based lipids play essential roles in the glycosylation process by delivering their bound glycan cargo to selected protein targets (46, 362). In *Archaea*, glucose-, mannose-, *N*-acetylglucosamine-, and sulfated tetrasaccharyl-containing phospho- and pyrophosphopolyisoprene (containing 11 to 12 isoprene units) were first observed in *Halobacterium salinarum* by ion exchange and thin-layer chromatography (281). Later studies (248) confirmed that the lipid moiety of these compounds is C₆₀ dodecaprenol. This is similar to the dolichol used in eucaryal protein N-glycosylation (46) but distinct from undecaprenol, which is composed of 11 unsaturated isoprene units and used by *Bacteria* for protein glycosylation and peptidoglycan synthesis (362, 425). Mass spectrometry and nuclear magnetic resonance-based approaches revealed the presence of *Eucarya*-like sugar carriers in *Haloferax volcanii*, including mannosyl-galactosyl-phosphodolichol, lesser quantities of a dihexosyl-phosphodolichol and a tetrasaccharyl-phosphodolichol containing mannose, galactose, and rhamnose, all linked to a dolichol containing 11 or 12 isoprene units (242).

(i) Antibiotics that affect dolichol processing interfere with archaeal protein glycosylation. The use of various antibiotics and other compounds known to prevent protein glycosylation by interfering with the processing of dolichol carriers has provided insight into the role of this lipid in archaeal protein N-glycosylation. Tunicamycin hinders transfer of UDP-*N*-acetylglucosamine to polysaccharide-loaded dolichol carriers (105). Treatment with this antibiotic interferes with *Sulfolobus acidocaldarius* S-layer glycoprotein glycosylation (147). In contrast, tunicamycin has no effect on the biosynthesis of the *Haloferax volcanii* S-layer glycoprotein (99) and accordingly, the glycan moiety of the *Haloferax volcanii* S-layer glycoprotein does not include *N*-acetylglucosamine (242, 280). Bacitracin is another drug that interferes with protein glycosylation via an interruption of the recycling of pyrophosphate-containing dolichol species (420). Accordingly, in *Halobacterium salinarum*, bacitracin interferes with the attachment of the repeating sulfated pentasaccharide found at the Asn-2 position of the S-layer glycoprotein (284, 469), although not with the attachment of the sulfated polysaccharide found at the other N-glycosylation sites of the protein (469).

Bacitracin also inhibits glycosylation of flagellins in *Methanococcus deltae* (27) and slowed *Sulfolobus acidocaldarius* growth, possibly through interference with the protein N-glycosylation pathway (286). In contrast, bacitracin had no effect on the glycosylation of the S-layer glycoprotein or a second 98-kDa glycoprotein in *Haloferax volcanii* (99, 232). The failure of the antibiotic to prevent *Haloferax volcanii* glycoprotein biogenesis is likely related to the fact that, unlike *Halobacterium salinarum*, in which both monophosphate- and pyrophosphate-containing dolichol oligosaccharide carriers are present (247), only bacitracin-insensitive monophosphate-containing oligosaccharide-dolichol intermediates are found in *Haloferax volcanii* (242). Incorporation of glucose from UDP-glucose into *Haloferax volcanii* glycoproteins was, however, inhibited by amphomycin and two sugar nucleotide analogs, PP36 and PP55 (489), compounds reported to block transfer of nucleotide-conjugated sugars to phosphopolyisoprenols in *Eucarya* (201, 202, 336).

(ii) Analysis of dolichol-bound glycans. Evidence for the involvement of dolichol phosphate-linked oligosaccharides in archaeal protein N-glycosylation also comes from examination of the carrier-bound glycan moieties. The transfer of radio-labeled glucose from UDP-[³H]glucose to *Haloferax volcanii* glycoproteins proceeds through a glucose-containing phosphopolyisoprenol intermediate (489). The dolichol-linked sulfated polysaccharide moiety found in *Halobacterium salinarum* is identical to glycan moieties found on the S-layer glycoprotein and flagellin in this species (248, 470). On the other hand, the sulfated polysaccharide is methylated at the dolichol-linked stage, whereas no 3-*O*-methylglucose is detected in the protein-linked polysaccharide (249).

The importance of this transient methylation is illustrated by the detrimental effect of inhibiting *S*-adenosylmethionine-dependent methylation. Such treatment interfered with glycoprotein biosynthesis but did not affect either general protein biogenesis or the biosynthesis of sulfated phosphodolichol-bound oligosaccharides. It thus appears that methylation is an essential step in the biosynthesis of the sulfated oligosaccharide moiety prior to being transferred to its nascent polypeptide target. By contrast, the hexasaccharide moiety attached to the *Methanothermobacter feravidus* S-layer glycoprotein retains its methylation (204). It is not clear whether such methylation is involved in the translocation of the sulfated oligosaccharide phosphodolichol across the membrane or the subsequent transfer of the glycan moiety to the nascent polypeptide chain. In *Eucarya*, chemical modification of glycoprotein glycan moieties occurs only after the oligosaccharide has been transferred to the nascent polypeptide (449).

Enzymes of N-glycosylation. Just as archaeal N-glycosylation relies on the dolichol carriers implicated in eucaryal protein glycosylation, *Archaea* also contain homologues of many of the enzymes involved in eucaryal N-glycosylation. These include those involved in oligosaccharide charging of the lipid carrier, translocation of the dolichol carrier across the membrane, and transfer of the oligosaccharide entity to the nascent polypeptide chain (Fig. 2).

(i) Genomic studies. Analysis of the NCBI protein database (www.ncbi.nlm.nih.gov) reveals the presence of genes encoding homologues of the staurosporine- and temperature-sensitive yeast protein 3 (Stt3p) (425), an essential protein thought to contain the active site of the multisubunit yeast oligosaccharide transferase complex (309, 493), in 18 archaeal strains. In *Bacteria*, such as *Campylobacter jejuni*, it is believed that the Stt3p homologue PglB is the only component needed for transfer of glycans to Asn residues during protein N-glycosylation (425).

A close examination of the *Archaeoglobus fulgidus* genome sequence revealed genes encoding STT3-like proteins within two gene clusters encoding putative homologues of other enzymes involved in yeast protein glycosylation (Fig. 3) (46). One of these clusters contains three adjacent open reading frames (ORFs), one of which encodes a polypeptide that appears to contain a motif present in the yeast Alg1p and Alg2p glycosyltransferase proteins. In the yeast proteins, this motif is involved in the transfer of nucleotide sugars to the phosphodolichol carrier (46). The other two ORFs putatively encode a dolichyl-phosphoglucose synthase homologue and a homologue of Stt3p. Other ORFs in this cluster show high sequence

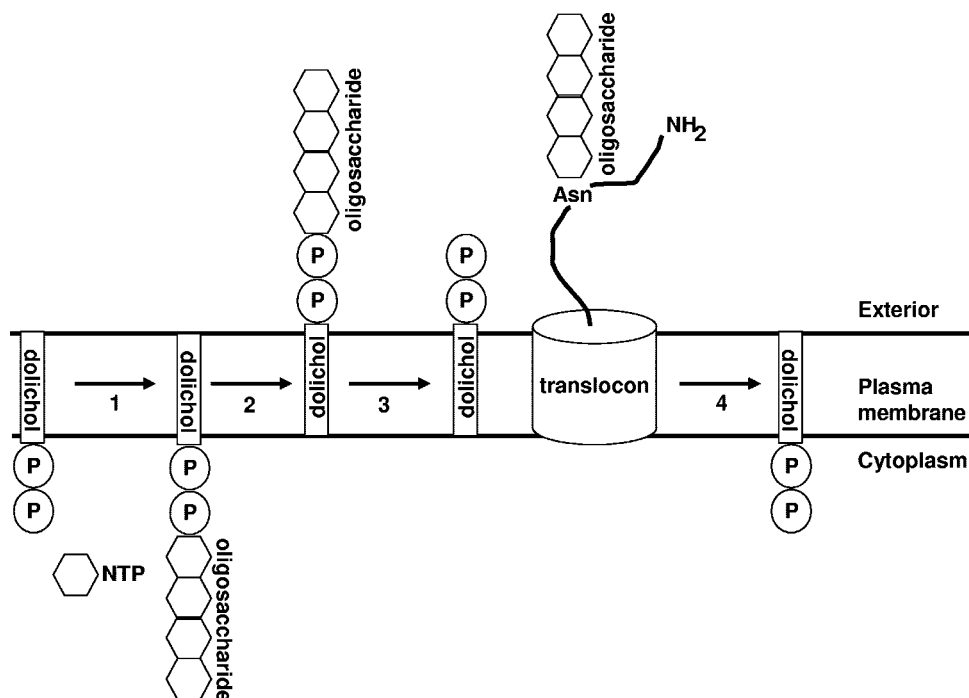


FIG. 2. Schematic depiction of archaeal N-glycosylation. Step 1. A dolichol pyrophosphate (or monophosphate) species is glycosylated by transfer of saccharide subunits from nucleotide sugars (or possibly from lipid-bound sugar precursors). Step 2. Glycosylated phosphodolichol “flips” across the plasma membrane, likely in an enzyme-mediated process. Step 3. The oligosaccharide structure is transferred to selected Asn residues of a newly translocated polypeptide. The figure does not consider the relationship between protein translation and protein translocation or the relationship between protein translocation and protein glycosylation. Step 4. Following transfer of the oligosaccharide moiety to a protein target, the phosphorylated dolichol carrier is recycled to its original topology. See references 247, 420, and 468, the text, and Table 3 for additional information.

similarity to RfbA and RfbB, components of a transporter family presumably involved in the flipping of bacterial O-antigen (467) and lipopolysaccharides (364) across the plasma membrane. While the functions of these putative gene products remain to be shown, it has been postulated that this *Archaeoglobus fulgidus* gene cluster encodes a functional unit involved in the assembly, translocation, and transfer of dolichol-phosphate-linked oligosaccharides to protein targets (46). The second gene cluster in *Archaeoglobus fulgidus* includes

ORFs also encoding putative glycosyltransferase, dolichyl-phosphoglucose synthase, and STT3 proteins, and lies near six ORFs bearing similarity to genes encoding proteins involved in bacterial lipopolysaccharide biosynthesis (46).

(ii) **Biochemical studies.** In addition to such gene-based predictions, enzymatic activity has also been demonstrated for some archaeal glycosylation-related proteins. Biochemical characterization of *Pyrococcus furiosus* UDP- α -D-glucose pyrophosphorylase, responsible for UDP-glucose synthesis, rep-

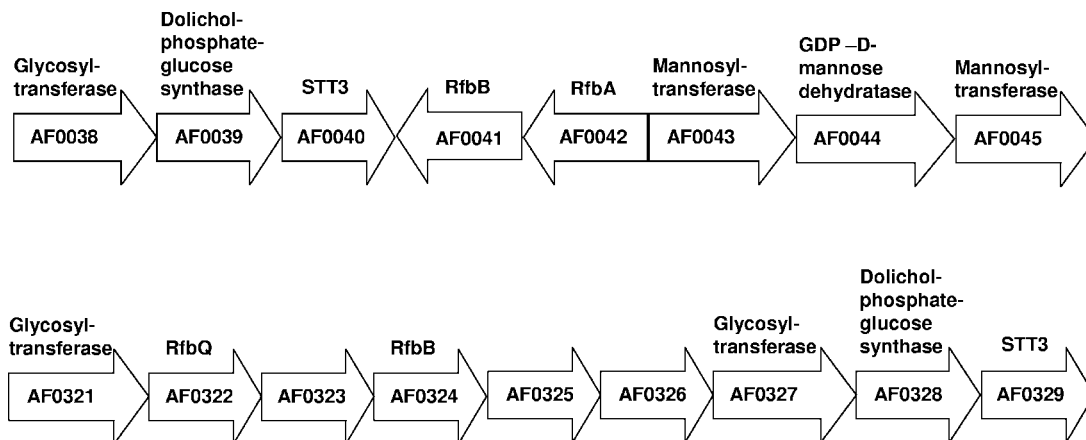


FIG. 3. Schematic depiction of two *Archaeoglobus fulgidus* gene clusters putatively involved in protein glycosylation. Putative gene products are given above each ORF. For further details, see reference 46.

resents the first analysis of an archaeal sugar nucleotidyltransferase (290). An *N*-acetylglucosamine transferase was also partially characterized from membranes of *Halobacterium salinarum* (281). Dolichylphosphate mannose synthase, which is able to transfer GDP-mannose to a dolichol phosphate carrier, was purified from *Thermoplasma acidophilum* (490). Amphomycin, an inhibitor of dolichylphosphate mannose synthases (202), blocked the activity of the enzyme (490). Using 5-azido-[³²P]UDP-glucose in a photoaffinity approach, a single 45-kDa species was identified in *Haloflex volcanii* homogenates that is thought to correspond to dolichylphosphate glucose synthase (489).

Pyrophosphatases with their active site oriented towards the cell exterior have been purified from the membranes of two different *Sulfolobus acidocaldarius* strains (8, 286). The pyrophosphate-hydrolyzing activity of the enzymes, proposed to participate in the hydrolysis of dolicholpyrophosphate-linked oligosaccharides during protein glycosylation, was stimulated in the presence of *Sulfolobus* membrane lipids. Sequence analysis of one of these pyrophosphatases has led to the identification of putative homologues in the genome sequences of *Sulfolobus tokodaii* and *Sulfolobus solfataricus* as well as in *Methanobacterium thermoautotrophicum* (294). This study also revealed the presence of a strongly conserved phosphatase tripartite sequence motif, Lys-XXXXX-Arg-Pro-X₁₂₋₅₄-Pro-Ser-Gly-His-X₃₁₋₅₄-Ser-Arg-XXXXX-His-XXX-Asp, also detected in Lpp1p and Dpp1p, *Saccharomyces cerevisiae* proteins showing hydrolytic activity towards dolichylphosphate, dolichylpyrophosphate, and other isoprenoid phosphates/pyrophosphates (116).

Subcellular localization of glycosylation. Several lines of evidence suggest that archaeal glycosylation occurs at the outer cell surface, the topological equivalent of the luminal-facing leaflet of the endoplasmic reticulum membrane bilayer, the site of N-glycosylation in *Eucarya* (46, 157, 235, 333, 409, 442). Despite its inability to cross the plasma membrane of haloarchaea (284), bacitracin is nonetheless able to interfere with *Halobacterium salinarum* protein glycosylation by preventing transfer of sulfated oligosaccharides to the S-layer glycoprotein (284, 469). The external orientation of the archaeal glycosylation apparatus is further supported by the decoration of exogenously added, soluble cell-impermeable hexapeptides containing the Asn-based N-glycosylation motif with sulfated oligosaccharides by living *Halobacterium salinarum* cells (248). Other observations also favor the assignment of archaeal protein glycosylation to the cell's outer surface. These include the ecto-enzymatic nature of a *Sulfolobus acidocaldarius* pyrophosphatase (8, 286), the proposed specific inhibition of an externally oriented Mg²⁺-dependent oligosaccharidetransferase by EDTA, a non-cell-permeant reagent, and subsequent interference with *Halobacterium salinarum* flagellin glycosylation (420), as well as studies supporting the cotranslational mode of membrane protein insertion in *Archaea* (360).

Role of Protein Glycosylation in Archaea

Structural roles. Given the seemingly routine glycosylation of archaeal proteins, one can ask what role is played by this posttranslational modification in *Archaea*. The observation that bacitracin treatment transformed rod-shaped *Halobacte-*

rium salinarum cells into spheres led to the proposed structural function of archaeal protein glycosylation (282). In fitting with a role for the sulfated S-layer glycoprotein oligosaccharide chains in maintaining the rod shape of *Halobacterium salinarum* cells, it was noted that similarities exist in the overall structures of the S-layer glycoprotein and proteoglycans, components of the extracellular matrix of animal cells (30, 468). For example, iduronic acid, a major component of proteoglycans (296), is found in the glycans decorating the *Halobacterium salinarum* S-layer glycoprotein. Similarly, the O-glycosylation cluster situated near the membrane-spanning base of the *Haloflex volcanii* S-layer glycoprotein has also been assigned a structural support role in the formation of a periplasmic-like space (217). In *Thermoplasma acidophilum*, an organism that lacks a cell wall, it has been suggested that the glycan moieties attached to the major glycosylated membrane-bound protein species coating the cell surface act to either trap water molecules or allow the cell surface proteins to interact with each other. In either scenario, glycosylation would contribute to the rigidity of the cell surface (478).

Functional roles. The glycosylation of archaeal proteins has also been implicated in protein assembly and function. In archaeal flagellins, glycosylation is associated with proper flagellar assembly, since upon bacitracin-mediated interference with flagellin glycosylation, a loss of *Methanococcus deltae* flagellation was observed microscopically (196). In a mutant *Halobacterium salinarum* strain in which underglycosylated flagellins are overproduced, increased levels of flagella were detected in the growth medium, suggesting proper flagellin glycosylation to be important for correct flagellar incorporation into the plasma membrane (470). This explanation is, however, inconsistent with the apparent nonglycosylated nature of other archaeal flagellins (184) or the glycosylation of *Methanospirillum hungatei* flagellins, which only occurs in low-phosphate media (406). Similarly, evidence against glycosylation's playing a role in protein function comes from bacterial expression of archaeal binding proteins. Normally glycosylated in their native hosts, nonglycosylated heterologously expressed versions of these proteins were also capable of substrate binding (170, 230, 231). Nevertheless, glycosylation could play a role in stabilization against proteolysis or could affect the interaction of binding proteins with the cell membrane or envelope (4).

Glycosylation as an environmental adaptation. Coping with the often harsh environmental conditions encountered by *Archaea* serves as the basis for yet another hypothesized role for archaeal protein glycosylation. In a comparison of the glycosylation profiles of S-layer glycoproteins from the moderate halophile *Haloflex volcanii* and the extreme halophile *Halobacterium salinarum*, it was noted that the latter experiences a higher degree of glycosylation than the former (280). Moreover, the glycan moieties of the extreme halophile were enriched in sulfated glucuronic acid subunits as opposed to the neutral sugars found in the moderate halophile. These properties endow the *Halobacterium salinarum* S-layer glycoprotein with a drastically increased surface charge density relative to its *Haloflex volcanii* counterpart.

The enhanced negative surface charges are thought to contribute to the stability of haloarchaeal proteins in the face of molar salt concentrations (266). Accordingly, the *Halobacterium salinarum* S-layer glycoprotein also contains 20% more

acidic amino acid residues than does the corresponding protein in *Haloflex volcanii* (246, 421). The enhanced negative surface charge associated with protein glycosylation and the resulting protection that this would afford in the face of acidic conditions have been offered as the role of *Sulfolobus acidocaldarius* cytochrome *b*_{558/566} glycosylation (161, 484). It has also been suggested that a significant amount of the protein surface is shielded from the ~pH 2 environment by the high degree of glycosylation (484). Finally, glycosylation has also been implicated in the stabilization of thermophilic archaeal glycoproteins (4, 258, 455).

LIPID MODIFICATION

Lipid modification, defined herein as the permanent or temporary covalent attachment of lipid-based groups at various positions within a polypeptide chain, is a common modification experienced by both eucaryal and bacterial proteins. An examination of known lipid modifications reveals that a wide variety of lipid moieties can be directly or indirectly linked to a protein at any of numerous attachment sites through the use of any of several linkages (414). For instance, lipid modification can involve myristoyl or palmitoyl acyl groups (358), isoprenyl polymers of various lengths (393), or aminoglycan-linked phospholipids (103). These can be added at the amino terminus, the carboxy terminus, or at internal residues via ester, thioester, thioether, or amide bonds, or through mediating elements, such as the phosphopantethene group of the acyl carrier protein (267).

Lipid modification of proteins is largely a posttranslational event (115). It serves a variety of roles, the most obvious being to enhance the membrane affinity of the modified protein. Accordingly, amino-terminal acylation leads to the localization of numerous proteins to the outer membrane of gram-negative *Bacteria* (156, 379), as exemplified by Braun's lipoprotein in *Escherichia coli* (40). Similarly, otherwise soluble eucaryal proteins also become membrane associated upon the covalent attachment of one or more lipid moieties (102, 153, 194, 462). Lipid modification can also modulate protein-protein interactions in *Eucarya*, as shown by the effects of myristylation or prenylation upon trimeric G protein subunit affinity (124, 178, 462), and in viruses, exemplified by the involvement of myristylation of the capsid proteins of human immunodeficiency virus type 1 and picornavirus in virion particle assembly and secretion (65, 142).

Lipid modifications of eucaryal proteins has also been implicated in a variety of other cellular events. These include signal transduction (287), embryogenesis and pattern formation (271), protein trafficking through the secretory pathway (297), and evasion of the immune response by infectious parasites (369, 461). Yet another role for lipid modification is exemplified by the bacterial toxin hemolysin A, which requires fatty acid acylation on an internal Lys residue for its activation (414).

Given the ubiquitous distribution and numerous functions of lipid modifications in eucaryal and bacterial proteins, it is not surprising that lipid-modified proteins have also been identified in *Archaea*.

Membrane Lipids of *Archaea*

One of the defining traits of *Archaea* that distinguish them from *Eucarya* and *Bacteria* is the chemical composition of their membrane phospholipids (206, 208). First, unlike eucaryal and bacterial phospholipids which are built on a glycerol-3-phosphate backbone, archaeal phospholipids are based on the opposite stereoisomer, glycerol-1-phosphate. Second, archaeal phospholipids contain polyisoprenyl side chains rather than the acyl groups employed by eucaryal and bacterial phospholipids. Third, archaeal phospholipids rely on ether bonds to link the isoprenyl side chains to the glycerol-1-phosphate backbone. In *Eucarya* and *Bacteria*, ester bonds link acyl side chains to the glycerol-3-phosphate backbone. Of these three traits, the use of glycerol-1-phosphate is considered the most defining, since examples of ether-linked lipids have been observed in *Eucarya* and *Bacteria* (172, 328) and non-ester-linked phospholipid fatty acids and genes encoding components involved in the metabolism of fatty acids have been reported in *Archaea* (127, 342). Indeed, free fatty acids have been observed in the lipid phase of *Methanospaera stadtmanae* and *Pyrococcus furiosus* (51, 191). Finally, archaeal phospholipids are generally organized into the bilayer structure that is also present in eucaryal and bacterial cells, although tetraether lipid-based monolayers can be found in thermophilic and hyperthermophilic *Archaea* (92, 226).

Whereas phospholipids and other polar lipids (phosphoglycolipids, glycolipids, and sulfolipids) account for the vast majority of archaeal membrane lipids, archaeal membranes also contain acetone-soluble nonpolar lipid species, primarily neutral squalenes and other isoprenoid-based polymers (206, 207, 334, 439, 440). In halophilic *Archaea*, in which membrane lipid composition has been most studied, pigmented carotenoids, in particular bacterioruberins, are major components of the nonpolar lipid pool (243, 438). These have been implicated in affording protection from UV-induced damage (390). In addition, many halophilic *Archaea* also contain retinal as part of bacteriorhodopsin, the purple retinal-containing protein complex that functions as a light-driven proton pump (244).

Lipid-Modified Archaeal Proteins

In *Archaea*, lipid-modified proteins have been reported from a wide range of species. In many cases, modification involves uncharacterized lipid entities, whereas in others, direct proof for the presence of attached lipid groups remains lacking. Table 4 summarizes the various lipid-based modifications shown or presumed to exist in *Archaea*, while Fig. 4 offers a schematic presentation of representative archaeal lipid-modified proteins.

Lipoproteins. In the haloalkaliphile *Natronobacterium pharaonis*, halocyanin, a small blue copper protein, was proposed to undergo amino-terminal lipid modification based on the presence of the so-called lipobox sequence motif near the start of predicted amino acid sequence (274). In *Bacteria*, the Leu-Ala-Gly-Cys lipobox sequence motif (156) lies at the end of the signal sequence, the short N-terminal extension not found in the mature, lipid-modified protein (see below). At the membrane, the bacterial lipobox motif is sequentially recognized and processed by three enzymes. The sulfhydryl group of

TABLE 4. Lipid modifications observed and proposed in *Archaea*

Modification	Species	Observed or predicted ^a	Reference(s)
N-terminally linked lipid (lipoprotein)	<i>Archaeoglobus fulgidus</i>	Predicted	4
	<i>Halobacterium salinarum</i>	Predicted	228
	<i>Halobacterium</i> sp. strain NRC-1	Predicted	4
	<i>Methanococcus jannaschii</i>	Predicted	4
	<i>Methanosarcina acetivorans</i>	Predicted	4
	<i>Methanosarcina mazei</i>	Predicted	4
	<i>Natronobacterium pharaonis</i>	Predicted	274
	<i>Pyrococcus abyssi</i>	Predicted	4
	<i>Pyrococcus furiosus</i>	Predicted	4
	<i>Pyrococcus horikoshii</i>	Predicted	4
	<i>Thermococcus litoralis</i>	Predicted	170
Isoprenylation	<i>Halobacterium cutirubrum</i>	Observed	376
	<i>Halobacterium salinarum</i>	Observed	218, 376
	<i>Haloferax volcanii</i>	Observed	233
Acylation	<i>Halobacterium cutirubrum</i>	Observed	350
	<i>Methanobacterium thermoautotrophicum</i>	Observed	350
GPI anchor	<i>Sulfolobus acidocaldarius</i>	Observed	224
	<i>Methanosarcina barkeri</i>	Predicted	310

^a See text for details.

the Cys residue is first modified with a diacylglyceride by pro-lipoprotein diacylglyceryl transferase, after which the upstream Gly-Cys bond is cleaved by signal peptidase II. The newly exposed N-terminal Cys residue of the protein then undergoes

additional acylation by apolipoprotein *N*-acyltransferase to yield the mature, lipid-modified lipoprotein (379). Direct proof for such modification of halocyanin has not been provided since the amino-terminal sequence of the protein could not be determined, possibly due to modification of the amino-terminal residue. Support for lipid modification of *Natronobacterium pharaonis* halocyanin, however, extends beyond the presence of the lipobox motif. Halocyanin is predicted to contain a β -turn after the lipobox, a structural feature that is characteristic of bacterial lipoproteins (130). Furthermore, mass spectroscopic analysis of halocyanin was consistent with the presence of two C₂₀ phytanyl groups ether linked to a glyceryl group (274).

In gram-positive bacteria, it is accepted that substrate-binding proteins, components of multisubunit ABC transporters responsible for cellular uptake of substrates, are lipoproteins (131, 422, 430). The same may well be true in *Archaea*. The trehalose/maltose-binding protein of the hyperthermophile *Thermococcus litoralis* contains a lipobox-like sequence motif and requires detergent for its solubilization (170). Similar motifs have been identified in other ABC sugar transporter binding proteins identified in *Archaea*, suggesting that amino-terminal lipid modification of binding proteins takes place in other species (4, 228).

Lipid modification is not, however, the sole mode of membrane association for archaeal sugar-binding proteins. For example, a membrane-spanning domain is predicted to anchor the glucose-binding protein of *Sulfolobus solfataricus* (3). It should be noted, however, that binding proteins in this organism differ from those in other *Archaea* in terms of amino-terminal sequence and subsequent posttranslational processing (see below). In *Halobacterium salinarum*, BasB and CosB, the first examples of binding proteins involved in chemotaxis in *Archaea*, are also thought to be lipoproteins due to their membrane localization and bearing of the lipobox sequence motif (228). Indeed, sequence analysis of putative substrate-binding proteins in *Halobacterium salinarum*, be they involved in nu-

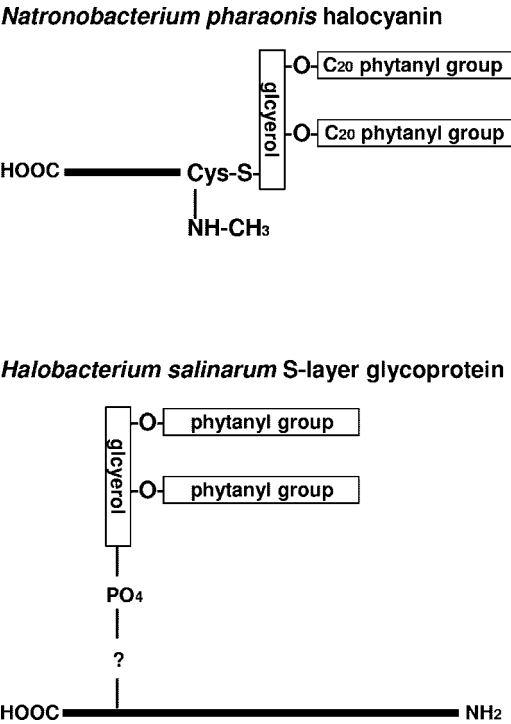


FIG. 4. Schematic depiction of representative archaeal lipid-modified proteins. Shown are *Natronobacterium pharaonis* halocyanin and *Halobacterium salinarum* S-layer glycoprotein. The lipid modification and acetylation of the amino-terminal Cys of *Natronobacterium pharaonis* halocyanin have not been experimentally proven, nor has the linkage or exact position of the diphytanylglycerolphosphate group found within the Thr-rich carboxy-terminal region of the *Halobacterium salinarum* S-layer glycoprotein. See text for details.

trient uptake or chemotaxis, suggests that all are lipoproteins (228). Finally, in the case of *Pyrococcus* species peptide-binding proteins, a conserved Gly-Cys motif reminiscent of the lipobox sequence located near the carboxy terminus may also be a target for lipid modification (4).

Despite the proposed presence of lipoproteins in *Archaea*, no archaeal homologue of signal peptidase II, one of the enzymes involved in lipoprotein precursor maturation, has been observed. Whether this is because there is no such enzyme in *Archaea* or because its sequence differs beyond recognition from that of its bacterial homologues, possibly in adaptation to the ether-based phospholipids of the archaeal membrane, remains unknown.

Isoprenylated proteins. Growth of *Halobacterium cutirubrum*, *Halobacterium salinarum*, and *Haloferax volcanii* in the presence of radiolabeled mevalonate, a precursor of the isoprene building block used to synthesize archaeal lipids (38, 398), led to the appearance of several proteins radiolabeled through the covalent attachment of a lipid entity (233, 376). Subsequent chemical analysis of the modifying lipid moiety in *Halobacterium salinarum* revealed a novel diphytanylglycerol methyl unit, linked to Cys residues of the modified proteins by a thioetheric bond (376). Further analysis of isoprenoid-modified proteins in *Halobacterium salinarum* using other radiolabeled isoprenyl derivatives revealed that the S-layer glycoprotein is modified by a second novel group, diphytanylglycerol phosphate, which is attached through an as yet uncharacterized linkage (218). Amino acid sequencing places the modification near an O-glycosylated Thr-rich stretch found in the C-terminal region of the protein, upstream of the single transmembrane domain (218). In *Haloferax volcanii*, lipid modification of the S-layer glycoprotein was also shown, although the chemical composition of the attached lipid is unknown, as is the site of attachment (99, 233).

Since haloarchaeal S-layer glycoproteins include a membrane-spanning domain (246, 421, 457), it is unclear why an additional membrane anchor in the form of a lipid would be required. Nonetheless, the attachment of the lipid moiety that takes place on the external surface of *Haloferax volcanii* and *Halobacterium salinarum* cells is responsible for the posttranslational, posttranslocational maturation of the S-layer glycoprotein in these strains, as detected through pulse-chase radiolabeling studies (99, 233). Furthermore, since other haloarchaeal S-layer glycoproteins also contain a sequence similar to that modified in *Halobacterium salinarum* (246, 421, 457), it would appear that such isoprenoid-based lipid modification of S-layer glycoproteins is a general trait of halophilic *Archaea* (218).

Acylated proteins. Since some *Archaea* contain significant amounts of fatty acids (51, 127, 191) and completed archaeal genome sequences reveal the presence of genes involved in fatty acid biosynthesis and β -oxidation (342), it should not come as a surprise that the acylation of archaeal proteins has been reported. In *Halobacterium cutirubrum* and *Methanobacterium thermoautotrophicum*, subcellular fractionation and analytical chemical techniques were employed to show the acylation of several proteins (350). Chromatographic analyses identified palmitic and stearic acids as the main modifying agents, although lower levels of modification by myristic acid and other fatty acids were also observed. These acyl groups are thought to be linked to the protein via amide or ester bonds.

GPI-anchored proteins. Glycosylphosphatidylinositol (GPI) anchors represent a carboxy-terminal posttranslational lipid-based modification used to tether eucaryal proteins to various membranes (176). The GPI anchor is added to target proteins using a preformed GPI-anchoring moiety which consists of a molecule of phosphatidylinositol linked at its myoinositol headgroup to ethanolamine phosphate through an aminoglycan bridge. This lipid is transferred to the newly exposed carboxy terminus of a nascent polypeptide. The modified protein is first synthesized as a membrane-anchored precursor that undergoes proteolytic processing upstream of its carboxy-terminal transmembrane domain. The cleaved protein is thus attached to the ethanolamine end of the preassembled GPI moiety.

Although widespread in the eucaryal domain, GPI-anchored proteins have not been observed in *Bacteria* (103). They have, however, been detected in *Archaea*. In *Sulfolobus acidocaldarius*, three proteins were identified that incorporate radiolabeled precursors of the GPI anchor moiety (224). One of these, a 185-kDa species, was also solubilized by the actions of a bacterial phosphatidylinositol-specific phospholipase C, a characteristic of GPI-anchored proteins (175). Although the other two *Sulfolobus* proteins were not released by the phospholipase, this is not inconsistent with GPI anchoring as phosphatidylinositol-specific phospholipase C-resistant GPI-anchored proteins have been reported (122, 365). Similarly, a typical archaeal ether-based phospholipid bearing the identical GPI anchor moiety head group as found in *Eucarya* was identified in *Methanosarcina barkeri* (310). Incubation of this lipid species with phosphatidylinositol-specific phospholipase C led to the release of the polar head group.

In addition to these biochemical studies, a bioinformatic analysis of available archaeal genome sequences predicts the presence of GPI-anchored proteins in other archaeal species (103). Moreover, many of the 19 enzymes known to participate in the biosynthesis of GPI anchors have been detected in archaeal genome sequences (104).

PROTEIN PHOSPHORYLATION

Like other forms of posttranslational modification considered in this review, the covalent attachment of phosphate groups to protein targets at any of a number of surface Asp, His, Ser, Thr, or Tyr residues can profoundly affect protein behavior. However, in contrast to N-glycosylation and, in most cases, lipid modification, covalent modification of proteins by phosphorylation is a reversible event. This property, combined with the major perturbation in protein structure that results from phosphorylation (189), has made this versatile form of posttranslational modification widely used when rapid and profound changes in protein behavior are called for (214, 215). As such, protein phosphorylation and dephosphorylation are most commonly exploited by the cell in adaptive pathways designed to present appropriate responses to various cues associated with a multitude of external and internal stimuli (173).

Although discovered in the 1950s (240), it took approximately 25 years for the first reports of phosphorylated proteins in *Bacteria* to appear (126, 459). Shortly thereafter, in 1980, the presence of phosphorylated proteins in *Halobacterium salinarum* was reported (413), confirming that *Archaea* too are ca-

TABLE 5. Archaeal proteins reported to be phosphorylated

Protein	Species	Phosphorylated residue	Evidence for phosphorylation	Reference
CheA	<i>Halobacterium salinarum</i>	His	³² P incorporation	374
CheY		Asp	³² P incorporation	374
Cdc6	<i>Methanobacterium thermoautotrophicum</i>	Ser	³² P incorporation	144
	<i>Pyrobaculum aerophilum</i>	Ser	³² P incorporation	144
	<i>Sulfolobus solfataricus</i>	Ser	³² P incorporation	89
aIF2 α	<i>Pyrococcus horikoshii</i>	Ser	³² P incorporation	426
Phenylalanyl-tRNA synthetase β -chain	<i>Thermococcus kodakaraensis</i> KOD1	Tyr	Antiphosphotyrosine antibodies	188
Phosphomannomutase	<i>Thermococcus kodakaraensis</i> KOD1	Tyr	Antiphosphotyrosine antibodies	188
RtcB	<i>Thermococcus kodakaraensis</i> KOD1	Tyr	Antiphosphotyrosine antibodies	188
Zinc-dependent aminopeptidase	<i>Sulfolobus solfataricus</i>		³² P incorporation	73

pable of performing this posttranslational modification. With the subsequent availability of genome sequences, it became clear that *Archaea* also contain numerous kinases and phosphatases, enzymes responsible for protein phosphorylation and dephosphorylation, respectively (214, 215, 253).

Targets and Functions of Protein Phosphorylation in *Archaea*

The first examples of archaeal protein phosphorylation were reported when *Halobacterium salinarum* grown in the presence of ³²P-labeled orthophosphate was shown to phosphorylate Ser and Thr residues of several protein species (413). The radiolabeling of 100- and 80-kDa proteins and, as shown later, an additional 62-kDa species (411) was, however, greatly diminished upon exposure to light. Moreover, the light-dependent dephosphorylation of these proteins could be linked to the proton motive force generated by the light-driven proton pump bacteriorhodopsin. In related studies (395), it was shown that growth in ³²P-labeled orthophosphate-containing growth medium led to the appearance of serine- and threonine-phosphorylated proteins of 71, 52, 42, and 31.5 kDa in *Sulfolobus acidocaldarius*, in a growth-phase-dependent manner. Further examination revealed the existence of an additional 40-kDa *Sulfolobus acidocaldarius* phosphoprotein that was threonine-phosphorylated in the presence of [³²P]polyphosphate (396). The first phosphoprotein with a known function to be identified in *Archaea*, however, was the methyltransferase activation protein from *Methanosarcina barkeri*, a key enzyme involved in the metabolic transformation of carbon dioxide to methane (81).

Although other phosphorylated proteins have been identified in *Archaea* (475), the observed phosphorylation cannot usually be attributed to a regulated protein kinase (see below), but rather reflects phosphorylated intermediates that appear during an enzyme's catalytic cycle. Such enzymes apparently include the α subunit of succinyl-coenzyme A synthase in *Sulfolobus solfataricus* (403) and *Sulfolobus acidocaldarius* glycogen synthase (52, 397). Nevertheless, examples of regulated protein phosphorylation in *Archaea* have been reported (Table 5) and are discussed below.

Phosphorylation of components involved in signal transduction. Protein phosphorylation as part of an archaeal two-component signal transduction pathway was first shown for *Halobacterium salinarum* (373, 374). In *Bacteria* and a very

limited number of *Eucarya*, two-component signal transduction response pathways are responsible for the appropriate response of the cell to a wide range of environmental conditions (234, 332, 423). The conformational changes that result upon ligand binding to the extracellular portion of a transmembrane receptor are transduced into the cell, where they lead to the modulation of sensor (histidine kinase, see below) and response regulator proteins. Such modulations ultimately activate the transcription of genes encoding compensatory proteins or affect the motion of the microorganism via motility structures. Transduction of the ligand binding event to sensor and response regulator proteins is achieved via a cascade of phosphorylation reactions. Hence, the detection of phosphorylated *Halobacterium salinarum* CheA and CheY, well-characterized sensor and response regulator proteins, respectively (114, 423), pointed to the presence of a two-component system in *Archaea*, charged with responding to various chemotactic and phototactic stimuli (373, 374).

Protein phosphorylation in response to environmental change has also been observed in other archaeal species. Growth of *Sulfolobus acidocaldarius* in the presence of radiolabeled phosphate under limited-phosphate conditions revealed the existence of numerous phosphoproteins (319). In particular, the phosphorylation of a 36-kDa protein was augmented under phosphate starvation, hinting at a regulatory role in a cellular response pathway for this protein. In *Haloferax volcanii*, growth at elevated salt concentrations may lead to the appearance of several serine-phosphorylated proteins not detected during growth under optimal salt conditions (32). A threonine-phosphorylated 67-kDa membrane protein displaying serine kinase activity has been found in *Sulfolobus solfataricus*, although the pathway in which this protein participates remains to be defined (261, 264).

Phosphorylation of components involved in DNA replication, cell cycle regulation, and translation. In addition to playing a role in signal transduction, protein phosphorylation has also been implicated in eucaryal DNA replication, cell cycle regulation, and protein translation (313, 314, 349). Similar roles for protein phosphorylation have also been observed in *Archaea*. In *Methanobacterium thermoautotrophicum*, *Pyrobaculum aerophilum*, and *Sulfolobus solfataricus* (89, 144), DNA-dependent serine autophosphorylation has been reported for the Cdc6 protein, an initiator protein that fulfills an essential role in DNA replication and is known to be phos-

phorylated in *Eucarya* (182, 212). The autophosphorylation of Cdc6 proteins reveals similarities between the archaeal and eucaryal replication processes, even though domain-specific differences in Cdc6 autophosphorylation have been noted (144). Protein phosphorylation also takes place during both eucaryal and archaeal protein translation. In vitro studies addressing the heterotrimeric archaeal initiation factor 2 complex (aIF2) from *Pyrococcus horikoshii* showed that the aIF2 α subunit could be phosphorylated (426), as is the case for the parallel eucaryal eIF2 α subunit (93, 251).

Phosphorylation of other proteins. In other instances, archaeal phosphoproteins have been indentified in which the role of this posttranslational modification remains obscure. In *Sulfolobus solfataricus*, for example, a novel zinc-dependent aminopeptidase, originally isolated from cell lysates in complex with a chaperonin, was shown to be phosphorylated (73).

Finally, whereas the bulk of phosphorylated archaeal proteins experience modification of Asp, His, Ser, or Thr residues, it is now known that archaeal proteins can also undergo phosphorylation at Tyr residues. Using antiphosphotyrosine antibodies, tyrosine-phosphorylated proteins were first identified in cell extracts of *Haloferax volcanii*, *Methanosarcina thermophila*, and *Sulfolobus solfataricus* (401). In *Thermococcus kodakaraensis* KOD1, tyrosine-phosphorylated proteins recognized by antiphosphotyrosine antibodies were subsequently identified by N-terminal sequencing as RtcB, which is involved in RNA processing (128), the phenylalanyl-tRNA synthetase β -chain, and phosphomannomutase (188). Thus, long thought to be restricted to *Eucarya* (255) and later shown to occur also in *Bacteria* (77), proof for the existence of archaeal tyrosine phosphorylation shows this form of posttranslational modification to be ubiquitous across evolution (475).

Archaeal Protein Kinases and Phosphatases

In general, phosphorylated proteins do not contain readily recognizable sequence regions that allow their assignment as candidates for this posttranslational modification. In contrast, protein kinases and phosphatases, the enzymes responsible for the addition and removal, respectively, of orthophosphate groups from target proteins, contain conserved sequence motifs (213). Based on such motifs, protein kinases and phosphatases can be divided into several functional families (213). Thus, the availability of several archaeal genome sequences has allowed a catalogue of the potential protein kinases and phosphatases to be assembled (214, 215). A better understanding of the archaeal proteins should also provide insight into the relationship between eucaryal and bacterial kinases and phosphatases, which were once thought to be distinct (234, 253). For a more detailed examination of archaeal kinases and phosphatases, the reader is directed to a recent review of the subject (215).

Eucaryal protein kinases. Members of the eucaryal protein kinase superfamily, an evolutionarily conserved group of proteins sharing a common core, serve as the major providers of protein serine/threonine/tyrosine kinase activity in *Eucarya* (154). Long considered to be restricted to *Eucarya*, homologues of eucaryal protein kinases were subsequently reported in *Bacteria* and more recently detected in *Archaea* (214). Initially, searches of the then-available archaeal gene sequences

identified ORFs in *Methanococcus thermolithotrophicus*, *Methanococcus vannielii*, and *Methanococcus voltae* encoding proteins whose carboxy-terminal regions contain 9 of 11 subdomains associated with eucaryal protein kinases (400). In a later study (215), analysis of nine completed archaeal genomes revealed the presence of ORFs encoding polypeptides containing sequence motifs essential for eucaryal protein kinase activity in seven.

Gene-based studies of individual strains have also revealed the existence of eucaryal protein kinases in other *Archaea*, such as in *Haloferax volcanii* cells exposed to elevated salt levels, in which a salt-regulated gene putatively encoding a protein serine/threonine kinase was detected (32). Subsequent studies employing complete archaeal genome sequences, moreover, have expanded our knowledge of eucaryal protein kinases. In a comprehensive search based on a large number of completed genome sequences, including those of four *Archaea*, archaeal representatives of four novel putative protein kinase families were reported (253), such as the Rio1 family, comprising only archaeal and eucaryal members, or the ABC1 family, including only a single archaeal representative (from *Methanobacterium thermoautotrophicum*). Furthermore, the recent solution of the crystal structure of *Archaeoglobus fulgidus* Rio2 suggests that this protein defines a new family of protein kinases (245).

In addition to sequence-based analyses, archaeal homologues of eucaryal protein kinases have been examined at the protein level. Analysis of threonine-modified phosphoproteins in *Sulfolobus solfataricus* membranes following incubation with [γ - 32 P]ATP led to the identification of the protein encoded by ORF *sso0469* (264). Sequence analysis revealed the presence of eukaryotic protein kinase motifs, while biochemical characterization of a recombinant version of the encoded protein revealed its ability to phosphorylate Ser residues of exogenous polypeptides in vitro. Similarly, SsoPK2, the product of *Sulfolobus solfataricus* ORF *sso2387*, also contains sequence motifs found in eucaryal protein kinases (263). Moreover, a recombinant form of the protein was able to phosphorylate itself as well as various exogenous targets, relying on that part of the protein homologous to eucaryal protein kinases, as revealed by mutagenesis approaches (263).

Histidine kinases. Histidine kinases are elements of the two-component signal transduction pathway described above. In response to conformational changes experienced by upstream receptor-transducer teams, histidine kinase sensors use ATP to autophosphorylate His residues before transferring the phosphoryl group to Asp residues of downstream response regulators. The first example of an archaeal histidine kinase as part of a two-component system identified was *Halobacterium salinarum* CheA (373). A recombinant version of the haloarchaeal CheA histidine kinase was autophosphorylated upon addition of radiolabeled ATP and was subsequently able to transfer its phosphoryl group to an Asp residue of the *Halobacterium salinarum* CheY response regulator (374).

In later homology-based searches of nine completed archaeal genome sequences, histidine kinases were identified in four: *Archaeoglobus fulgidus*, *Halobacterium* sp. strain NRC-1, *Methanobacterium thermoautotrophicum*, and *Pyrococcus horikoshii* (215, 220, 234). Of these, *Methanobacterium thermoautotrophicum* and *Archaeoglobus fulgidus* contain the most histidine kinases (16 and 14, respectively) and response regulators

(10 and 11, respectively). At the other extreme, *Pyrococcus horikoshii* contains only a single histidine kinase and two response regulators (corresponding to CheA and to CheY and CheB, respectively), while *Aeropyrum pernix*, *Methanococcus jannaschii*, and *Thermoplasma acidophilum* are not predicted to encode such proteins. The absence of Che proteins in *Methanococcus jannaschii* is noteworthy, given that this species is both flagellated and motile (436).

Protein serine/threonine phosphatases. Protein serine/threonine phosphatases can be structurally and functionally grouped into the protein serine/threonine phosphatase (PPP) and the Mg^{2+} and Mn^{2+} protein phosphatase (PPM) families (21). PPP family members are mainly responsible for serine/threonine dephosphorylation in *Eucarya* and have also been reported in *Bacteria* (71, 213, 214). In contrast, members of the PPM family are the primary mediators of dephosphorylation in *Bacteria*, although this family encompasses several eucaryal protein phosphatase classes as well (37, 214). In *Archaea*, members of both protein serine/threonine phosphatase families have been identified in completed genome sequences and some have been studied at the protein level (213–215).

To date, three PPP family protein serine/threonine phosphatases have been characterized from *Archaea*. The genes encoding PP1-arch1, PP1-arch2, and Py-PP1 were cloned from *Sulfolobus solfataricus* (216, 252), *Methanosarcina thermophila* TM-1 (321, 403), and *Pyrodictium abyssi* TAG11 (268), respectively. In addition, other archaeal PPP family phosphorylases have been predicted following analysis of genome sequences, relying on the presence of conserved sequence motifs (24, 215). Such sequence comparisons revealed the archaeal enzymes to be more closely related to their eucaryal than their bacterial homologues (24). However, despite their sequence similarities to eucaryal PPP family members, archaeal PPP family protein serine/threonine phosphatases display a combination of eucaryal and bacterial features (215). Like their eucaryal counterparts, the archaeal enzymes specifically act upon protein-bound phosphoserine and phosphothreonine residues and, in the cases of PP1-arch2 and Py-PP1, are inhibited by toxic secondary metabolites such as okadaic acid (268, 321, 403). In contrast, the three archaeal PPP family members require the addition of metal ions such as Mn^{2+} for activity, as is the case for bacterial PPP family protein serine/threonine phosphatases (391). Finally, protein serine phosphatase activity has also been detected in extracts of *Halobacterium salinarum* (36) and *Haloferax volcanii* (320), but the enzymes responsible have not been identified.

A single ORF encoding a potential PPM family protein serine/threonine phosphatase was identified in the genome sequence of *Thermoplasma volcanium*. The putative protein includes all of the conserved sequence elements of PPM family members (209).

Protein tyrosine phosphatases. While ORFs thought to encode protein tyrosine phosphatases have been detected in *Archaeoglobus fulgidus*, *Methanococcus jannaschii*, *Methanobacterium thermoautotrophicum*, *Pyrococcus abyssi*, *Pyrococcus furiosus*, *Pyrococcus horikoshii*, *Sulfolobus solfataricus*, and *Thermococcus kodakaraensis* KOD1 (215, 418), only the *Thermococcus kodakaraensis* KOD1 enzyme has been examined biochemically (188). A recombinant version acted on both free

phosphotyrosine and phosphoserine, suggesting that it had dual specificity. Moreover, a mutant form of the enzyme was used to capture putative native substrates from a cell extract (188). In addition, studies performed with *Halobacterium salinarum* extracts detected protein serine/threonine phosphatase activity also able to hydrolyze phosphotyrosine, suggesting the responsible enzyme similarly had dual specificity (36).

Protein kinases and phosphatases of *Thermoplasma acidophilum*. It should be noted that analysis of the genome of *Thermoplasma acidophilum*, using tools available today, has failed to detect the presence of any protein kinase or phosphatase (214). While it remains to be seen whether the current inability to recognize such proteins will be remedied in future with the development of more powerful bioinformatic prediction tools, it is also possible that *Thermoplasma acidophilum* contains novel archaea-specific kinases or phosphatases, or does not perform protein phosphorylation. Interestingly, genome analysis of two bacterial strains, a *Buchnera* sp. and *Rickettsia prowazekii*, also failed to detect ORFs encoding putative protein kinases or phosphatases (214), although the implications of these studies are at present unknown.

PROTEIN METHYLATION

Although methylation of nucleic acids is well known, in part due to a role in disease states such as cancer (86, 256, 424), a wide variety of proteins have also been reported to experience posttranslational methylation. This modification affects the amino group in the side chains of Ala, Arg, Glu, His, Lys, and Pro residues, the hydroxyl group in the side chains of Glu and Asp, and the thiol group of Cys residues (327). Enzyme-catalyzed addition of methyl groups from *S*-adenosylmethionine can either occur reversibly, as in *O*-methylation of carboxyl groups, or irreversibly, as in the *N*-methylation of amino-terminal or side chain nitrogen atoms (70).

As is the case with other posttranslational modification events considered in this review, analysis of protein methylation in *Archaea* has revealed novel forms of protein methylation as well as providing new insights into the biological role served by this posttranslational modification.

Protein Methylation in Response to External Stimuli

As described above, various external stimuli that modulate the motility of archaeal cells rely on phosphorylation of elements of the two-component signal transduction response pathway. Phosphorylation is not, however, the sole posttranslational modification experienced by proteins involved in taxis responses to environmental cues. As in *Bacteria* (90, 229, 236, 423), numerous proteins involved in the archaeal response to growth conditions also undergo methylation (see below). Methylation of taxis receptor or transducer proteins is thought to be responsible for adaptation, a form of cellular memory necessary for cells to be able to sense and move towards ever higher attractant concentrations or to recognize when motion is occurring in the wrong direction, i.e., away from elevated attractant concentrations (423).

Three methylation-dependent taxis responses, phototaxis, chemotaxis, and aerotaxis, have been detected in *Halobacte-*

rium salinarum, in which the archaeal response to environmental cues, as mediated through transducer proteins, has been well studied. In *Halobacterium salinarum*, the phototactic response is initiated by the excitation of the two retinal-containing photoreceptors, sensory rhodopsin I and sensory rhodopsin II (121, 239, 341, 412, 482, 488). These subsequently relay the excitatory signal to their respective transducer proteins, HtrI and HtrII. During phototaxis, these proteins undergo methylation, a posttranslational modification previously shown to modulate the life span of phototactic signals in *Halobacterium salinarum*, i.e., to play an adaptative role (164). Methylation of HtrII is also involved in the transducer role assumed by the protein during serine chemotaxis (171). The cytoplasmic transducer HtrXI undergoes methylation/demethylation in response to changes in extracellular histidine, aspartate and glutamate concentrations (43).

Arginine taxis in *Halobacterium salinarum* involves the methylatable soluble transducer Car, which monitors intracellular levels of the amino acid (417), while the methylation status of the membrane-bound transducer BasT affects chemotactic behavior towards leucine, isoleucine, valine, methionine, and cysteine (227). HtpIV, or CosT, the transducer for the haloarchaeal chemotaxis response towards trimethylammonium compounds, also experiences methylation (228). The aerotactic (oxygen gradient-sensing) response of *Halobacterium salinarum* was also shown to rely on methylation, in this case of the membrane-bound transducer HtrVIII (259). In contrast, aerotaxis in *Bacteria* such as *Escherichia coli* and *Salmonella enterica* serovar Typhimurium does not require transducer methylation (259). Most recently, MpcT, the transducer of membrane potential changes in *Halobacterium salinarum* (formerly known as HtrXIV) was shown to experience differential degrees of methylation (225).

Methylation of Methyl-Coenzyme M Reductase

In methanarchaea, the final reaction in the release of methane is catalyzed by the enzyme methyl-coenzyme M reductase (434). Analysis of the crystal structure of the enzyme from *Methanobacterium thermoautotrophicum* revealed the presence of five modified amino acid residues in the α subunit of the hexameric enzyme, all situated near the active-site region (108). In addition to a thioglycine residue, the enzyme contains 1-*N*-methylhistidine, 5-(*S*)-methylarginine, 2-(*S*)-methylglutamine, and an *S*-methylcysteine residue (Fig. 5). Whereas 1-*N*-methylhistidine and *S*-methylcysteine have been detected in other proteins (70, 326) and a thiol-modified glycine residue has been identified in ThiS, one of the enzymes involved in thiamine biosynthesis in *Escherichia coli* (432), *Methanobacterium thermoautotrophicum* methyl-coenzyme M reductase is the first example of a 2-(*S*)-methylglutamine and 5-(*S*)-methylarginine. Previously, only *N*-methylglutamine and *N*-methylarginine had been reported (162, 492).

The posttranslational modifications leading to the appearance of the four methylated amino acids in methyl-coenzyme M reductase involve the transfer of the methyl group of methionine, most likely in the *S*-adenosylmethionine form (387). The modifications are thought to occur before methyl-coenzyme M reductase assumes its quaternary structure, since the modified residues are buried deep inside the native enzyme,

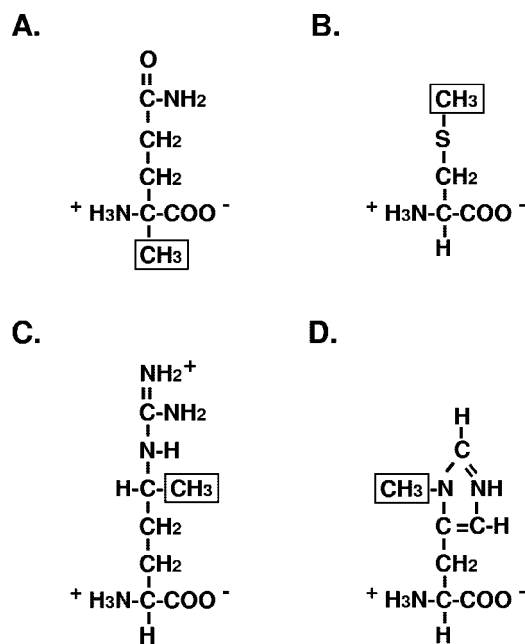


FIG. 5. Methylated amino acids in *Methanobacterium thermoautotrophicum* methyl-coenzyme M reductase. A. 2-(*S*)-Methylglutamine. B. *S*-Methylcysteine. C. 5-(*S*)-Methylarginine. D. 1-*N*-Methylhistidine. In each case, the modifying methyl group is boxed.

where they would be inaccessible to *S*-adenosylmethionine or methyltransferases, which catalyze protein methylation (108, 143). Furthermore, considering the differences in amino acid composition in the vicinities of the four methylated residues (387), it is probable that four different *S*-adenosylmethionine-dependent methyltransferases are involved in the modification reactions (308). Accordingly, multiple methyltransferases appear to be present in the genome sequence of *Methanobacterium thermoautotrophicum* (399).

In terms of function, methylation of His-257, which is involved in substrate binding, likely affects the substrate affinity of the enzyme (108). The thioglycine residue has been proposed to serve as a one-electron relay in the catalytic mechanism (434). The functional significance of the methylation of the other three modified residues, i.e., 5-(*S*)-methylarginine-271, 2-(*S*)-methylglutamine-400, and *S*-methylcysteine-452, remains unknown. However, analysis of methyl-coenzyme M reductase sequences in a wide range of methanarchaeal species reveals the absolute conservation of the five amino acid residues modified in the *Methanobacterium thermoautotrophicum* enzyme (311, 410). Moreover, the crystal structure of methyl-coenzyme M reductase from *Methanosarcina barkeri* also revealed the presence of thioglycine, *S*-methylcysteine, 1-*N*-methylhistidine, and 5-methylarginine residues, i.e., four of the five posttranslational modifications found in the *Methanobacterium thermoautotrophicum* enzyme, suggesting that such modifications are important for catalysis (143).

Methylated Proteins in Thermophilic Archaea

Methylated Lys residues have been detected in several thermophilic archaeal proteins, such as *Sulfolobus acidocaldarius*

ferredoxin (289) and *Sulfolobus solfataricus* glutamate dehydrogenase (272), aspartate aminotransferase (485), and β -glycosidase (117). In the case of *Sulfolobus solfataricus* β -glycosidase, *N*- ϵ -methylation of specific Lys residues was associated with increased thermal stability as well as with a lower susceptibility to denaturation and aggregation, in comparison to the nonmethylated recombinant version of the enzyme produced in *Escherichia coli* (117). The methylated Lys residues found in the *Sulfolobus solfataricus* enzyme are not conserved in other mesophilic glycosidases belonging to glycosyl hydrolase family I, again pointing to a thermostabilizing role for this posttranslational modification.

Methylation of Archaeal DNA-Binding Proteins

Although grouped with *Bacteria* as prokaryotes (472), *Archaea* resemble *Eucarya* in many aspects, including that members of both domains contain histones, proteins involved in DNA packaging (355, 466). First demonstrated in *Methanothermobacter fervidus* (378, 415), over 30 archaeal histone sequences have since been identified (355). Archaeal histones are, however, apparently restricted to *Euryarchaea*, an archaeal subdomain, in which several different histone-encoding genes have been detected (355, 466). No archaeal histones have been observed in *Crenarchaea*, the other major archaeal subdomain. Instead, crenarchaeal species contain small, basic DNA-binding proteins thought to fulfill the same functions as histones, based on their physical properties (63, 64, 149, 355, 367, 466). In *Sulfolobus*, these can be grouped into 7-, 8-, and 10-kDa classes, with the 7-kDa proteins, referred to as the Sul7 family (466), predominating. Members of the Sul7 family in both *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* are modified by monomethylation of selected Lys residues to different extents in a strain-dependent manner (26, 63, 64, 97, 278, 317).

Given the modulation of eucaryal histone function that results from methylation (59, 238), it is likely that methylation of archaeal Sul7 proteins also affects their behavior. Indeed, the observation that methylation of Sul7 proteins increased during heat shock suggests that such posttranslational modification is of functional, although as yet undefined, significance (26). Sul7 methylation does not, however, affect DNA binding affinity, consistent with the positioning of methylated Lys residues on the surface of the Sul7d-DNA crystal rather than at the protein-DNA interface (25). Finally, it is somewhat ironic that while archaeal Sul7 proteins are methylated, no evidence for methylation of archaeal histones has appeared, in contrast to their eucaryal counterparts (355). This is due to the fact that archaeal histones lack the amino- and carboxy-terminal extensions that undergo this posttranslational modification in eucaryal histones (59). Indeed, analysis of archaeal genome sequences reveals homologues of only one of the components involved in the eucaryal histone modification event, i.e., the histone acetyltransferase Elp3 (355).

Methylation of Archaeal Ribosomal Proteins

Several bacterial ribosomal proteins, mainly found in the large 50S subunit, undergo methylation (55, 56). Of these, L11 is the major methylated ribosomal component. Analysis of *Halobacterium cutirubrum* and *Sulfolobus solfataricus* L11 pro-

teins from cells grown in the presence of radiolabeled methionine and/or methylmethionine revealed that they are also methylated, albeit in a pattern distinct from that of the bacterial protein (9, 353, 354). Accordingly, genome searches have failed to identify an archaeal homologue of the bacterial L11 methyltransferase PrmA (45). The role of L11 methylation in both *Bacteria* and *Archaea* remains unknown.

DISULFIDE BONDS IN PROTEINS

In both *Eucarya* and *Bacteria*, secreted and extracellularly oriented membrane proteins are often stabilized by disulfide bonds, i.e., covalent links between the sulfhydryl groups of Cys residues in the same or different polypeptide chains. These can serve two roles. First, they can stabilize proteins by entropic destabilization of the unfolded conformation (78, 322, 463, 465). Second, they serve to limit damage to a protein resulting from oxidative or proteolytic agents, thereby enhancing protein lifetime. Accordingly, disulfide bonds are routinely employed by secretory and plasma membrane proteins in numerous organisms (315, 451).

The various compartments of the cell greatly differ in terms of redox potential and hence in their ability to catalyze disulfide bond formation. Accordingly, disulfide bond formation takes place in the endoplasmic reticulum of eucaryal cells (444) and in the periplasmic/extracellular compartment of bacterial cells (193, 351). In both locations, oxidative conditions favor disulfide bond formation and enzymes implicated in this posttranslational modification are found. Conversely, it had been generally accepted that proteins found in the reducing environment of the cytosol do not contain disulfide bonds, although it has recently become clear that a number of cytosolic proteins can contain specific and reversible disulfide bonds (see below). In such cases, the cyclic oxidation/reduction of a disulfide bond can control the activation/deactivation or otherwise modulate the activity of a protein (62, 80, 181, 325, 363). Indeed, controlled reduction of disulfide bonds has also been adopted by certain disulfide-containing secreted proteins and cell surface receptors (166). Nevertheless, the number of cytoplasmic proteins in *Eucarya* and *Bacteria* experimentally shown to contain disulfide bonds is limited. *Archaea*, however, do not follow this trend (269).

Disulfide Bonds in Cytoplasmic Archaeal Proteins

Unexpectedly, biochemical and structural characterization of many cytoplasmic archaeal proteins has revealed the presence of disulfide bonds. A disulfide bond was detected in *Pyrococcus furiosus* ferredoxin, in which it plays a role in the redox cycle of the protein (141). The crystal structures of DNA polymerases from *Thermococcus gorgonarius* and *Thermococcus* sp. strain 9°N-7 revealed the presence of two disulfide bridges in each case (169, 368). The recombinant form of *Aeropyrum pernix* alcohol dehydrogenase was shown to contain a disulfide bond (152) as was *Sulfolobus solfataricus* glyceraldehyde-3-phosphate dehydrogenase (180). The three-dimensional structure of the TATA box-binding protein from the hyperthermophile *Pyrococcus woesei* revealed the presence of a disulfide bond not found in mesophilic versions of the protein (88). Indeed, in many of these examples, the presence of di-

sulfide bonds is believed to contribute to the enhanced thermostability of the modified protein.

Disulfide bonds are also used by cytosolic archaeal proteins for the generation of higher-order structures. As revealed by X-ray crystallography and site-directed mutagenesis, a single intersubunit disulfide bridge is responsible for the dimeric nature of *Sulfolobus solfataricus* glycosyltrehalose trehalohydrolase (118) and pyrrolidone carboxyl peptidase from *Thermococcus litoralis* (394) and *Pyrococcus furiosus* (316). Similarly, ferric reductase from *Archaeoglobus fulgidus* was shown to be a homodimer, with a single disulfide bond serving to link the two subunits of the protein (60). In *Pyrococcus horikoshii*, oligomerization of isopropylmalate isomerase relies on intersubunit disulfide bridges (483). The homotetrameric structure of *Pyrococcus abyssi* tRNA (m1A) methyltransferase is also due to disulfide bonding (370). The nuclear magnetic resonance structure of *Pyrobaculum aerophilum* DsrC, the archaeal homologue of the γ subunit of dissimilatory sulfite reductase, responsible for the reduction of sulfite in sulfate-reducing bacteria, was also shown to contain two disulfide bonds (76). Disulfide bond formation is also responsible for the hexameric states of L-isoaspartyl-O-methyltransferase from *Sulfolobus tokodaii* (431) and of 5'-deoxy-5'-methylthioadenosine phosphorylase from *Sulfolobus solfataricus* (11, 47).

Despite the seemingly widespread presence of disulfide bonds in cytoplasmic archaeal proteins, it was only with the detection of three disulfide bonds in the crystal structure of *Pyrobaculum aerophilum* adenylosuccinate lyase (441) that the concept of the general use of disulfide bonds in cytoplasmic proteins in this and possibly other hyperthermophilic *Archaea* was proposed (269). Accordingly, computational analysis of completed archaeal genomic sequences, involving sequence-structure mapping approaches with subsequent analysis of the proximity of pairs of Cys residues, indicated that disulfide bonds are indeed prevalent in thermophilic and hyperthermophilic crenarchaeal cytoplasmic proteins, yet are not found in mesophilic versions of the same proteins (269). In this study, it was predicted that 44 and 40% of intracellular protein Cys residues in *Pyrobaculum aerophilum* and *Aeropyrum pernix* (both $T_{\text{opt}} \sim 100^\circ\text{C}$), respectively, and approximately 30% of the Cys residues in *Pyrococcus abyssi* and *Pyrococcus horikoshii* (both $T_{\text{opt}} \sim 100^\circ\text{C}$) cytoplasmic polypeptides are found in disulfide bonds. In *Archaeoglobus fulgidus* ($T_{\text{opt}} \sim 90^\circ\text{C}$), *Methanobacterium thermoautotrophicum* ($T_{\text{opt}} \sim 80^\circ\text{C}$), and *Methanococcus jannaschii* ($T_{\text{opt}} \sim 60^\circ\text{C}$), only 11 to 15% of the intracellular protein Cys content is predicted to participate in disulfide bonds. It thus appears that there exists a correlation between optimal growth temperature and the number of intracellular disulfide bond-containing proteins. Hence, disulfide bridge formation may well be one of many mechanisms known to enhance protein stability in *Archaea*. Interestingly, the same study (269) points to the presence of cytoplasmic disulfide bond-incorporating proteins in thermophilic *Bacteria* such as *Aquifex aeolicus* and *Thermotoga maritima*.

Disulfide Bonds in Extracellular Archaeal Proteins

The presence of disulfide bonds in archaeal secreted or membrane proteins has been reported in only a limited number of cases. Tetrabrachion, the major structural component of

the *Staphylothermus marinus* S-layer, was reported to contain disulfide bonds based on the destabilizing effect of dithiothreitol treatment in the face of thermal and proteolytic challenges (345). Disulfide bonds have also been postulated to be present in halolysin R4, a serine protease secreted by *Haloferax mediterranei*, since mutagenesis of either of two Cys residues in a carboxy-terminal extension of the protein or complete removal of this domain drastically reduced both the amount and activity of the heterologously expressed protein (198). One explanation offered for these observations was that a putative disulfide bond, linking the two Cys residues in question, would assume a stabilizing role in the native protein. Possible disulfide bond formation involving Cys residues in the S-layer glycoprotein of *Methanococcus jannaschii* has been also been offered as an explanation for the thermostability of this protein, relative to other methanococcal S-layer glycoproteins which do not contain Cys residues (1). Such predictions, however, await experimental verification.

Enzymes Involved in Disulfide Bond Formation in Archaea

The reduced nature of the cytoplasm of eucaryal and bacterial cells (174, 363) and the seeming abundance of disulfide-bonded intracellular proteins in thermophilic and hyperthermophilic *Archaea* (269) raise questions concerning the redox state of the archaeal cytoplasm and the nature of the proteins that are involved in disulfide bond formation in these organisms.

In eucaryal and bacterial cells, the formation and redox states of disulfide bonds are mediated by protein disulfide oxidoreductases (168). Members of this ubiquitous protein family, which includes thioredoxins, glutaredoxins, disulfide bond formation (Dsb) proteins, and protein disulfide isomerases (PDI), have active sites containing the Cys-X-X-Cys sequence motif and the thioredoxin fold structural motif (273). DsbA is found in the bacterial periplasmic space and is involved in protein disulfide bond formation (193, 351), while PDI catalyzes protein disulfide bond formation, reduction, and rearrangement in the eucaryal endoplasmic reticulum (444, 471). Acting as strong reductants in various cellular processes (120, 348), both the thioredoxin system, involving two thioredoxins and thioredoxin reductase, and the glutaredoxin system, including three glutaredoxins and glutathione reductase, maintain intracellular disulfide bonds in the reduced state through NADPH-dependent pathways (168, 363).

To date, few archaeal protein disulfide oxidoreductases have been described (see below) and, considering the limited information available, it is too early to assign any of them a physiological role. What is known, however, points to the unique character of the archaeal proteins. For instance, *Methanobacterium thermoautotrophicum* contains a small protein (Mt0807) with a thioredoxin/glutaredoxin-like fold that exhibits sequence similarity to glutaredoxins, including the characteristic Cys-Pro-Tyr-Cys active-site motif (279). While its function was initially tentative, subsequent structural analysis and sensitive enzyme assays (10) revealed it to be a true thioredoxin. Nuclear magnetic resonance-based structural studies of another *Methanobacterium thermoautotrophicum* protein (Mt0895) revealed that it too contains a thioredoxin/glutaredoxin-like fold. This protein was originally annotated as a conserved hypothet-

ical protein (31). The apparent absence of glutathione in *Archaea* (279, 301) together with the use of more precise structural analysis and activity assays led to the conclusion that Mt0895 is a thioredoxin. Structural and biochemical studies have shown that the same is true for *Methanococcus jannaschii* Mj0307 (54, 250). It has been suggested that proteins possessing a thioredoxin/glutaredoxin-like fold and a glutaredoxin-like active-site amino acid sequence but thioredoxin activity, such as Mt0895, Mt0807, and Mj0307, could belong to an ancient family predating the appearance of the present-day glutaredoxin and thioredoxin families that still exist in *Archaea* (10, 31).

As described above, the presence of disulfide bonds in non-cytoplasmic archaeal proteins remains to be conclusively proven. If this posttranslational modification is indeed employed by such proteins, one can ask whether the introduction of disulfide bonds involves archaeal homologues of PDI or the Dsb proteins, which are used by *Eucarya* and *Bacteria*, respectively, for this purpose (193, 444). The available information points to the presence of PDI-like proteins in *Archaea*. The structure of a protein disulfide oxidoreductase from *Pyrococcus furiosus*, originally predicted by sequence analysis to be a glutaredoxin-like protein (151), revealed the presence of two domains, each organized into the characteristic thioredoxin/glutaredoxin fold and both containing the Cys-X-X-Cys active-site motif (356). This is reminiscent of eucaryal PDI, which also contains two thioredoxin/glutaredoxin folds (85). By contrast, thioredoxin, glutaredoxin, and DsbA contain a single thioredoxin/glutaredoxin fold each (273).

Subsequent biochemical characterization of the *Pyrococcus furiosus* protein revealed that it, like eucaryal PDI, also displays oxidative, reductive, and disulfide isomerase activities (339). In addition, a homologous protein had been purified earlier from *Sulfolobus solfataricus* (150) and was predicted to exist in other species, based upon examination of the genome sequences of hyperthermophilic *Archaea* (339). However, the homologous protein from *Pyrococcus horikoshii* together with a second protein identified as a thioredoxin reductase were shown to function as a thioredoxin system, mediating electron transfer from a thioredoxin reductase-like flavoprotein to a protein disulfide bond, suggesting a role for this protein other than as a disulfide bond-introducing PDI (205).

PROTEOLYTICALLY PROCESSED PROTEINS

Posttranslational protein modification also includes proteolytic cleavage of precursor forms of proteins. In *Archaea*, examples of proteolytic processing at the amino and carboxy termini, in addition to positions within a polypeptide chain, have been reported.

Archaeal Signal Sequences

In any cell, a subset of proteins must cross one or more membranes to realize their ultimate localization and fulfill their designated roles. Across evolution, such proteins are generally synthesized with a cleavable amino-terminal extension referred to as the signal sequence that is enzymatically removed once such proteins have traversed the membrane. Analysis of signal sequence composition in *Archaea* as well as their

posttranslational removal reveals a mosaic of archaeal, eucaryal, and bacterial traits.

Protein translocation in *Archaea*. Translocation of extracytoplasmic proteins begins with their delivery to translocation sites in the membrane (42, 119, 298). Examples of both post- and cotranslational translocation have been found in *Archaea*. Chimeric signal sequence-bearing reporter proteins are secreted posttranslationally from transformed *Haloferax volcanii* cells (179). In addition, *Haloferax volcanii* has been reported to posttranslationally insert a chimeric protein containing the multispanning membrane protein bacterio-opsin (318). In contrast, cotranslational translocation, shown to be the general mode of membrane protein insertion in *Haloferax volcanii* (360), likely involves the archaeal signal recognition particle pathway (293, 494), as first reported for *Halobacterium salinarum* bacterio-opsin (83, 84, 148).

In *Archaea*, as across evolution, the Sec translocon is the major site for protein export (94). The SecY, SecE, and Sec61 β proteins that form the core of the translocation apparatus are closer to their eucaryal than their bacterial homologues (49, 155, 223, 347, 357, 361). The recent solution of the three-dimensional structure of the *Methanococcus jannaschii* SecYE β translocon has provided major insight into the translocation event across evolution, including the mode of translocon gating and mechanism of membrane protein insertion (446). The Sec translocon may also be involved in the translocation of archaeal flagellins, despite their distinct signal sequence composition (see below) (184, 436). In contrast to their bacterial counterparts, which cross the plasma membrane through the hollow core at the center of the growing flagellum (7, 265), archaeal flagellins are likely translocated across the membrane and only then added to the base of the growing motility structure, as gauged by the presence of unique cleavable N-terminal signal sequences in the archaeal proteins (20, 184, 436).

Archaea also use the twin-arginine targeting (Tat) pathway, a second protein export pathway (446). The Tat pathway can be distinguished from the Sec pathway by the unique composition of substrate signal sequences (see below) and by the ability of the Tat pathway to translocate folded or cofactor-incorporating proteins (29, 366). Although the Tat pathway is proposed to predominate in halophilic *Archaea* (35, 371), little is presently known of the workings of the Tat system in these or other organisms.

Genomic surveys of archaeal signal sequences. Descriptions of archaeal signal sequences have largely relied on analysis of genome sequences, using computer-based tools originally designed to detect eucaryal or bacterial signal sequences (2, 16, 35, 94, 306, 371, 377). At best, these algorithms should be able to identify only those archaeal signal sequences bearing sufficient similarity to their eucaryal and bacterial counterparts. Archaeal signal sequences possessing domain-specific traits would, therefore, likely be overlooked in such searches. Thus, true characterization of archaeal signal sequences will have to wait for the number of experimentally verified targets to be extended well beyond the few experimentally verified sequences presently available. Nonetheless, such efforts have identified signal sequences recognized by the Sec and Tat pathways, archaeal flagellin-like signal sequences on both flagellin and nonflagellin proteins, as well as lipoprotein signal se-

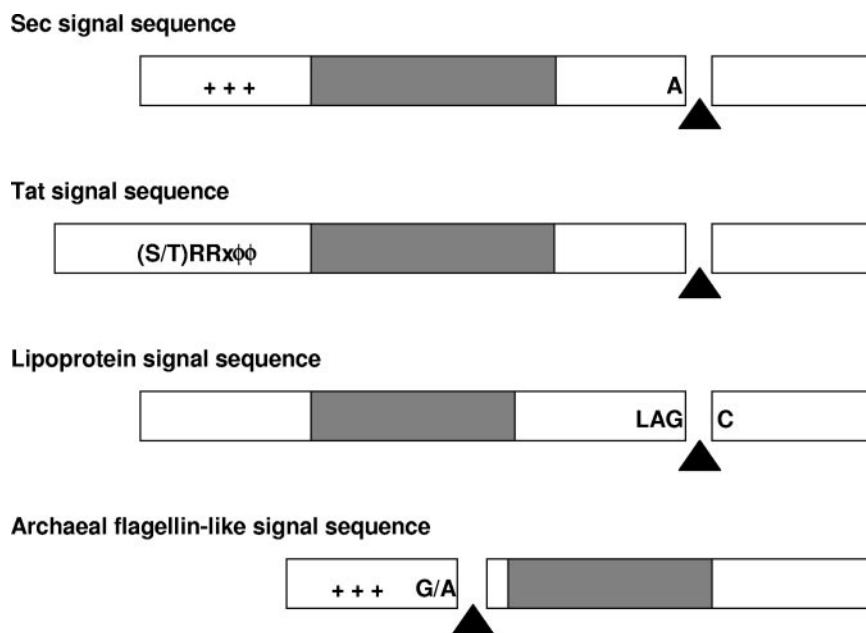


FIG. 6. Schematic depiction of archaeal signal sequences. In each case, consensus sequence elements characteristic of that class of signal sequence are shown, where + corresponds to positively charged residues, x corresponds to any residue, and ϕ corresponds to a hydrophobic residue. Hydrophobic stretches of amino acid residues are portrayed in gray. The site of cleavage is denoted by the black wedge.

quences (Fig. 6).

While the signal sequences of Sec pathway substrates can differ widely, they share common structural traits, such as a positively charged amino-terminal region leading to a hydrophobic core region that continues into an uncharged polar region terminating in the signal peptidase cleavage site (454). From examination of 10 genome sequences, it was concluded that predicted archaeal Sec signal sequences are more similar to their bacterial than their eucaryal counterparts (16, 307). The findings of this multigenome study (16) are in agreement with earlier studies addressing predicted signal sequences in *Methanococcus jannaschii* (306) and *Sulfolobus solfataricus* (2), although differences exist. Nonetheless, as discussed below, apparent similarities in the mechanism of archaeal and eucaryal signal peptidase action (18, 100, 437) suggest similarities between the cleavage site regions of signal sequences in these two domains.

While sharing the same tripartite organization, Tat pathway signal sequences differ from those recognized by the Sec pathway in that the former include an extended amino-terminal region containing a highly conserved motif based on two Arg residues and a less hydrophobic core region (29, 366). Analysis of genome sequences predicts limited-use presence of Tat signal sequences in *Archaea* (2, 16, 94), with the apparent exception of halophilic *Archaea* (35, 371). Here, proteins bearing Tat signal sequences are predicted to greatly outnumber those synthesized with Sec signal sequences. The enhanced utilization of the Tat pathway by halophilic *Archaea* is thought to be a response to the highly saline cytoplasm in these strains, reportedly as high as 5 M (67, 132). It has been postulated that to overcome dangers to protein folding associated with maintaining a "loosely folded" conformation in a high-salt environ-

ment, as would be required for posttranslational translocation by the Sec pathway, reliance on the Tat pathway, capable of translocating folded protein substrates, is preferable.

In addition to Sec and Tat pathway signal sequences, archaeal proteins may be synthesized as precursors bearing other cleavable signal sequences. As first noted in *Methanococcus voltae* (113), archaeal flagellins are made as precursors bearing atypical short, positively charged signal sequences, reminiscent of signal sequences found in bacterial type IV prepilins (20, 184, 436). Unexpectedly, genome analysis predicted the presence of the same signal sequence in a set of 10 extracellular *Sulfolobus solfataricus* proteins, including six putative solute-binding proteins (2). Archaeal flagellin signal sequences have also been predicted to exist in other types of protein, including those assigned solute-binding roles, in *Methanococcus jannaschii*, *Pyrococcus horikoshii*, *Sulfolobus shibatae*, and *Thermococcus litoralis* (5). In contrast to Sec and Tat pathway signal sequences, cleavage of archaeal flagellin signal sequences by the appropriate signal peptidase (see below) occurs upstream, rather than downstream, of the hydrophobic core region.

As discussed above, sequence analysis studies have also predicted the existence of proteins synthesized with lipoprotein signal sequences in *Archaea* (4, 170, 228, 274), although experimental support for these predictions has yet to be presented.

Removal of archaeal signal sequences. The signal sequences of proteins translocated by either the Sec or Tat pathway are removed by the actions of type I signal peptidases (82, 324). In *Archaea*, type I signal peptidases incorporate traits of both their eucaryal and bacterial counterparts. As in *Eucarya*, the archaeal signal peptidase does not rely on the catalytic Ser-Lys dyad employed by the bacterial enzyme and has replaced the conserved bacterial Lys with a His residue (100, 324, 437). At

present, the catalytic mechanisms of both archaeal and eucaryal signal peptidases remain largely undefined (18, 447). On the other hand, in contrast to the eucaryal enzyme, which functions as part of a larger signal peptidase complex (477), both bacterial and archaeal signal peptidases apparently function independently (100, 324). Furthermore, certain archaeal signal peptidases incorporate a sequence domain of unknown function, referred to as domain II (323). This domain is found in the bacterial but not the eucaryal enzyme (100).

The limited experimental analysis of archaeal signal peptidase activity available comes from studies in which the gene encoding the enzyme from *Methanococcus voltae* was heterologously expressed in *Escherichia coli* (302). Isolated bacterial membranes then served as the source of the archaeal enzyme in an in vitro signal peptidase assay, using a truncated, poly-His-tagged version of the *Methanococcus voltae* S-layer protein as the substrate. Site-directed mutagenesis of the *Methanococcus voltae* enzyme identified three conserved residues, Ser-52, His-122, and Asp-148, essential for activity (18). The finding that a second conserved Asp residue (Asp-142) was not crucial for catalytic function suggests differences in the mechanisms of the archaeal and eucaryal signal peptidases, since Asp residues found at both corresponding positions are essential for activity of the *Saccharomyces cerevisiae* enzyme (324).

Type II signal peptidases are involved in the removal of signal sequences from lipoproteins (156). However, as noted above, no archaeal type II signal peptidase has been described, despite the apparent existence of archaeal lipoproteins (4, 170, 228, 274).

The unique signal sequences of archaeal flagellins are removed by the actions of a signal peptidase reminiscent of bacterial type IV prepilin peptidases (17, 75), exemplified by *Pseudomonas aeruginosa* PilD (419). Those translocated non-flagellar *Sulfolobus solfataricus* proteins bearing the archaeal flagellin signal sequence also rely on an archaeal version of the bacterial type IV prepilin peptidase, termed PibD (peptidase involved in biogenesis of prepilin-like proteins), for their processing (2, 6). Site-directed mutagenesis studies have begun to provide insight into the catalytic mechanism of the archaeal enzyme (6, 17).

Amino-Terminal Methionine Removal

In many instances, the initiator Met residue of a nascent polypeptide chain (or *N*-formyl-Met residue in *Bacteria*) is cleaved by the actions of methionine aminopeptidases (39, 137). While the reason for such processing remains unclear, several explanations, including facilitation of additional amino-terminal processing (13) and modulation of protein lifetime (39, 450), have been suggested. Indeed, methionine aminopeptidases are essential for the survival of *Bacteria* and yeasts (57, 257).

Methionine aminopeptidases are cobalt-dependent enzymes that can be divided into two groups, based on sequence comparison (14, 210). Type I methionine aminopeptidases are found in *Eucarya* and *Bacteria*, although the eucaryal enzyme includes an amino-terminal extension not found in its bacterial counterpart. *Eucarya* also contain a second isoform of the enzyme, referred to as type II methionine aminopeptidases. The two enzyme classes can be distinguished by the presence of an

additional ~60-amino-acid-residue carboxy-terminal stretch of unknown function in type II enzymes (14). Genome sequence analysis has revealed that *Archaea* contain only type II methionine aminopeptidases, although these lack an amino-terminal extension found in the eucaryal enzyme (427, 443). Examination of the crystal structure of the *Pyrococcus furiosus* enzyme confirmed the similarities of the catalytic domains of the two methionine aminopeptidase isoforms, despite their limited degree of sequence homology (74, 427).

Inteins in Archaeal Proteins

Inteins are genetic elements lying within a protein-encoding ORF that are transcribed and translated together with their host to yield an immature precursor protein (135, 260, 337). Self-splicing of inteins occurs at the posttranslational level, when the inteins excise themselves from the host protein, allowing the intein-bordering residues of the flanking segments of the host polypeptide to join through a peptide bond to yield the mature protein, which is now able to fold and function properly. Although first discovered in a yeast vacuolar ATPase (200) and found in proteins across evolution, inteins are most commonly observed in archaeal proteins; by spring 2005, approximately 200 inteins had been reported, with almost half being found in *Archaea* (references 343 and 346 and databases cited therein).

Inteins are most often found in enzymes involved in DNA replication and repair. Indeed, the first archaeal intein was found in a *Thermococcus litoralis* DNA polymerase (344). Inteins have subsequently been detected in numerous other archaeal DNA polymerases (48, 305, 408, 429, 476) but also in other proteins (68, 110, 359, 388) from various hyperthermophilic *Archaea*. This list includes *Methanobacterium thermoautotrophicum* ribonucleoside diphosphate reductase, which contains the smallest known intein to date (110). Examination of intein databases reveals that archaeal inteins are not restricted to hyperthermophilic proteins but are also predicted to exist in proteins from acidophiles such as *Ferroplasma acidarmanus*, *Ferroplasma acidiphilum*, and *Picrophilus torridus*, from the haloarchaea *Halobacterium* sp. strain NRC-1, *Haloferax volcanii*, and *Haloarcula marismortui* and from the Antarctica-derived methanogen *Methanococcoides burtonii* (343, 346).

Understanding the mechanics of the self-splicing reaction associated with intein excision began with experiments employing hyperthermophilic archaeal DNA polymerases (312, 337, 476). In fitting with the elevated growth temperatures of the host organism, intein splicing from these proteins occurs inefficiently at temperatures below 25°C. By inserting the coding sequence of the intein from *Pyrococcus* sp. strain GB-D DNA polymerase between genes encoding two foreign proteins, an intein-containing chimeric precursor was expressed in *Escherichia coli* at low temperatures (476). Subsequent splicing of the purified precursor could be initiated by raising the temperature. Such studies revealed that all the information needed for the splicing process is found within the sequences of the intein and flanking regions and that the excision reaction is catalyzed by the intein itself, without need for additional factors.

Since these pioneering studies, further examination of archaeal inteins has revealed the diversity of intein biochemistry

and offered additional insight into this posttranslational modification. For instance, during the first of four steps involved in the intein self-splicing reaction, the conserved intein amino-terminal Ser or Cys residue undergoes an acyl rearrangement, resulting in the formation of a (thio)ester bond at the amino-terminal splice junction (312, 337). A *Methanococcus jannaschii* ATPase provided the first example of an intein bearing an amino-terminal Ala residue (140), leading to the description of an alternative splicing pathway (407). In the third step of the self-splicing reaction, cyclization of the intein carboxy-terminal Asn residue leads to peptide bond cleavage and subsequent excision of the intein (312, 337). This cyclization step is facilitated by the intein's penultimate His residue, which renders the carboxy-terminal Asn residue's carbonyl carbon more electrophilic (312, 337). Examination of intein cleavage from *Methanococcus jannaschii* phosphoenolpyruvate synthase and RNA polymerase subunit A' has provided insight into how inteins lacking this penultimate His residue are processed (58). Furthermore, the presence of inteins in DNA polymerases from *Halobacterium* sp. strain NRC-1, *Pyrococcus abyssi*, and *Pyrococcus horikoshii* bearing carboxy-terminal Gln rather than Asn residues suggests that inteins may self-excise via mechanisms not involving side chain cyclization (58). Indeed, dissection of the self-splicing pathway of the *Pyrococcus abyssi* DNA polymerase II DP2 subunit intein failed to detect the formation of an intein intermediate containing a cyclized C-terminal Glu residue (288).

Carboxy-Terminal Maturation of Archaeal [NiFe] Hydrogenases

Examination of the *Methanococcus voltae* *vhuU* gene product, a component of a [NiFe] hydrogenase, revealed that the translated polypeptide was shorter than predicted by the gene sequence due to a carboxy-terminal cleavage of the protein (404, 405). Although first demonstrated in *Methanococcus voltae*, cleavage of a carboxy-terminal region downstream of an Asp-Pro-Cys-X-X-His sequence motif by a dedicated endopeptidase following nickel incorporation has since been shown to be a general feature of prokaryotic [NiFe] hydrogenases (53). Differences in the [NiFe] hydrogenase proteolytic maturation step do exist, however, between the bacterial and archaeal systems. In *Escherichia coli* and other *Bacteria*, the hydrogenase cleavage motif is followed by a stretch of 15 or more amino acid residues (53). By contrast, in the proteolytic processing of the *Thermococcus kodakaraensis* hydrogenase α subunit, only four amino acid residues were released from the carboxy terminus following the conserved cleavage motif (199).

Similarly short sequences are also thought to be released from the large subunit of hydrogenases in other archaeal strains, including *Methanobacterium thermoautotrophicum* (380), *Pyrococcus furiosus* (352), and *Thermococcus litoralis* (433). Moreover, in *Methanobacterium thermoautotrophicum* and *Pyrococcus furiosus*, the mature enzymes are proposed to terminate in an Arg rather than a His residue. Differences between predicted molecular weight and the smaller molecular mass measured by SDS-PAGE migration suggest that proteolytic maturation of the *Pyrococcus furiosus* enzymes does indeed occur (380, 433).

In EchE, the *Methanosarcina barkeri* homologue of the *Esch-*

erichia coli hydrogenase 3 large subunit, the cleavage motif also terminates with an Arg residue, although in this case, proteolytic processing does not occur and the Arg thus corresponds to the terminal residue of the protein (241). In contrast, the homologous proteins in *Methanococcus jannaschii* and *Methanobacterium thermoautotrophicum* also have Arg rather than His residues at this position and yet contain carboxy-terminal extensions that likely undergo proteolytic processing (241). Finally, although the maturation process experienced by archaeal hydrogenases has been less well characterized than the parallel process in *Bacteria*, archaeal homologues of bacterial enzymes involved in this posttranslational maturation process have been described (199, 428).

OTHER POSTTRANSLATIONAL MODIFICATIONS IN ARCHAEA

Protein Acetylation

The acetylation of selected Lys residues in a protein was first observed almost 40 years ago with eucaryal histones (129), in which this posttranslational modification acts to modulate transcription (474). Since then, acetylation has been reported to modulate the function of many eucaryal and a limited number of bacterial proteins (237, 416, 479). In *Archaea*, protein acetylation of so-called Alba proteins has been demonstrated by mass spectrometry. These are a family of small (10 kDa) DNA binding proteins first detected in *Sulfolobus* species (28, 96). They have since been identified in numerous other eucaryal and crenarchaeal species as well as in *Eucarya* (28, 458, 460, 466). Upon acetylation of *Sulfolobus solfataricus* Alba at the Lys-16 position, the affinity of the protein for DNA was lowered (28). In vitro experiments support a role for the *Sulfolobus* homologue of the eucaryotic histone deacetylase Sir2 in deacetylating Alba, although other deacetylases may act similarly (466).

The enzyme responsible for Alba acetylation has not been identified, although several possible candidates are evident in archaeal genome sequences (355, 466). In a second case, the amino terminus of halocyanin, the small blue copper protein from the haloalkaliphile *Natronobacterium pharaonis*, has also been proposed to undergo acetylation, in addition to lipid modification (see above), based on the results of mass spectroscopic studies (274).

Protein Ubiquitination

The proteasome is a multisubunit complex responsible for protein degradation in the cytoplasm of eucaryal (452) and archaeal (91, 276, 277) cells. In *Eucarya*, proteins are targeted for proteasomal degradation by the posttranslational covalent attachment of ubiquitin, a 76-amino-acid-residue polypeptide (69). At present, the putative posttranslational modification that leads to proteasome-mediated protein degradation in *Archaea* has not been defined. While some reports suggest the existence of ubiquitin in *Archaea* (275, 300, 473), no direct demonstration of archaeal ubiquitin has been provided, nor have analyses of archaeal genomes identified ubiquitin-encoding genes or genes encoding ubiquitin-transferring proteins. Structural studies, however, have revealed the existence of ar-

chaical proteins bearing ubiquitin-like folds (33, 375). Nonetheless, it remains to be shown that these proteins participate in proteasome-mediated protein degradation in *Archaea*.

Hypusine-Containing Archaeal Protein

Hypusine [*N*- ϵ -(4-amino-2-hydroxybutyl)-L-lysine] is a non-standard amino acid residue found in all *Eucarya* in a single protein, eukaryotic translation initiation factor 5A (eIF-5A) (329). Hypusine is irreversibly formed soon after the biogenesis of eIF-5A in a two-step posttranslational reaction (331). In the first step, catalyzed by deoxyhypusine synthase, the 4-aminobutyl moiety of the polyamine spermidine is transferred to the ϵ -amino group of a single specific Lys residue in the eIF-5A precursor protein to form an intermediate, deoxyhypusine. The 4-aminobutyl group of the intermediate undergoes hydroxylation by deoxyhypusine hydroxylase to yield hypusine. The presence of this hypusine residue is essential for eIF-5A function (50, 330, 331).

Hypusine also exists in *Archaea*, where it too is found exclusively in aIF-5A, the archaeal homologue of eIF-5A. This has been shown in *Halobacterium cutirubrum*, *Methanococcus jannaschii*, *Pyrobaculum aerophilum*, *Pyrococcus horikoshii*, *Sulfolobus acidocaldarius*, and *Thermoplasma acidophilum* (22, 221, 338, 386, 480). The ability to synthesize hypusine has been studied in *Acidianus ambivalens*, *Pyrodicticum occultum*, and *Thermoproteus tenax* (23). The involvement of aIF-5A in archaeal cell growth and the archaeal cell cycle was shown by the arresting action of *N*¹-guanyl-1,7-diaminoheptane, a hypusination inhibitor, in *Halobacterium salinarum*, *Haloferax mediterranei*, *Sulfolobus acidocaldarius*, and *Sulfolobus solfataricus* (183).

PROTEOME-WIDE ANALYSIS OF POSTTRANSLATIONAL MODIFICATIONS IN ARCHAEA

Most studies of posttranslational modification of archaeal proteins have thus far relied on individual genes or proteins, the choice of which has been largely guided by substrate availability. In the future, one can expect that our ever-improving ability to describe the entire genomic, transcriptomic, and proteomic profile of an organism will move the study of posttranslational protein modification from the scale of selected proteins to a cellwide perspective. At present, however, such attempts are limited by various factors, including the possible heterogeneity of posttranslational modifications experienced by a given gene product, the relative abundance of a given posttranslationally modified protein variant, and our ability to discern potential posttranslational modifications not encountered previously. Nevertheless, as better tools become available for the simultaneous analysis of the entire protein complement of a cell (187, 270), proteomewide description of posttranslational modifications will soon become routine.

To date, several *Archaea* have been the subject of proteomic analysis. Such studies have provided novel insights into the adaptations adopted by extremophilic *Archaea* or have described technical advances in working with extremophilic proteomes (34, 66, 109, 123, 133, 134, 138, 167, 187, 192, 203, 295, 304, 491). Of these investigations, a number have focused on

posttranslational modification of archaeal protein targets. In the first of a series of studies addressing the proteome of *Methanococcus jannaschii*, the appearance of a given gene product in multiple positions in a two-dimensional gel electrophoretic system was taken to reflect the posttranslational modification of that polypeptide (134). Accordingly, the multiple positions of Mj0324, which is annotated as an elongation factor (EF-1 α), were assumed to correspond to isoforms modified by various degrees of phosphorylation, as had been observed with the eucaryal version of the protein (160). Mj0822, which is annotated as the S-layer glycoprotein, was also found in multiple positions in two-dimensional gels (134). In fact, protein spots corresponding to Mj0822 are among the most strongly stained by Coomassie blue, although the protein is resistant to silver staining, probably due to the presence of glycan moieties. Such differential staining of glycosylated proteins is well documented (186).

In a subsequent proteomic analysis of *Methanococcus jannaschii* as a function of growth conditions or growth stage, examination of peptide fragments derived from either Mj0891 or Mj0891, which are annotated as flagellin B1 and flagellin B2, respectively, revealed condition-specific changes in isoelectric point and abundance (133). Such modulations were proposed but not shown to result from differential degrees of posttranslational modification of the proteins.

More recent studies relying on advances in mass spectrometry for proteome analysis, which included elimination of intermediate proteolytic steps, resulted in a 100% sequence coverage of a set of 72 *Methanococcus jannaschii* proteins (123). Among these proteins, examples of protein acetylation and methylation, amino-terminal proteolytic processing, and disulfide bonds were detected. The applicability of this approach for the rapid determination of expected posttranslational modifications was shown when it was used to test the validity of histone acetylation in *Methanosarcina acetivorans* (125). Despite the proposed presence of a histone-modifying enzyme in this species, no histone modification was detected.

CONCLUSIONS

Archaea have proven to be a valuable resource in the search for new information on posttranslational protein modification. In several cases, *Archaea* have provided the first prokaryotic examples of modifications once thought to be restricted to *Eucarya*. The glycosylation of the *Halobacterium salinarum* S-layer glycoprotein represents one such example. In other cases, *Archaea* present previously unknown variations on a given posttranslational protein modification theme, such as the methylation profile of methyl-coenzyme M reductase or the unique lipid moieties attached to haloarchaeal proteins. Elucidation of the enzymatic steps involved in the archaeal version of a particular posttranslational modification event, such as signal sequence cleavage or intein splicing, has dramatically enhanced our understanding of the mechanistics of many posttranslational modifications.

With the advent of the proteomic era, when one can determine the protein profile of individual cells, complete physiological systems, and even entire organisms in response to a myriad of conditions, the protein complexity arising from posttranslational modifications should become even more obvious.

If past contributions are any indication, the study of *Archaea* will continue to expand understanding of the scope, the roles, and the biogenesis of posttranslational modifications that a protein can experience. Such information could provide insight into the strategies adopted by *Archaea* in the face of the extreme environments in which they can exist. One immediate benefit of relating posttranslational protein modifications to protein structure, stability, and function, together with enhanced tools for the manipulation of archaeal protein expression and secretion, will be the utilization of enzymes from extremophilic *Archaea* tailored for a broad spectrum of biotechnological and industrial applications.

ACKNOWLEDGMENTS

This work was supported by the Israel Science Foundation-Charles H. Revson Foundation (grant 433/03 to J.E.) and the National Science Foundation (BES-0317911 and MCB 0129841 to M.A.).

We thank Frank E. Jenney, Jr., for critical reading of the manuscript and the two anonymous reviewers for valuable comments and suggestions.

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